UNIVERSITY COLLEGE LONDON
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Identification and evaluation of biomarkers for Huntington's disease

A THESIS
SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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For Marc

Declaration of authorship and originality

I, Edward John Wild, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Edward John Wild

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Abstract

Huntington's disease (HD) is a devastating, incurable inherited neurodegenerative disorder that commonly affects adults in mid-life. Despite encouraging results from in vitro and animal trials, disease-modifying therapeutic trials in HD are limited by a lack of tools to track disease progression. HD is clinically heterogeneous, and current clinical rating scales lack sensitivity and specificity, particularly over relatively short time periods. Improvements in the precision of objective measurement of disease progression in HD could lead to state markers (biomarkers) better able to predict onset, detect progression and measure the effects of therapeutic intervention. Biomarkers capable of detecting disease-related changes in premanifest gene carriers will be essential for clinical trials of treatments to delay onset. Imaging, clinical and cognitive assessment as well as laboratory markers have all been proposed as biomarkers, but few measures have been quantified over short time intervals or shown to be predictive of clinical change over longer periods.

A robust panel of biomarkers from a number of modalities will be necessary to progress to interventional clinical trials of disease-modifying therapies in HD, using biomarkers to measure the success or failure of an intervention. Such cross-validation requires simultaneous multimodal biomarker evaluation within a suitable cohort of subjects studied longitudinally.

This thesis describes a multi-modal approach to the discovery and evaluation of potential biomarkers for Huntington's disease in a large cohort of human volunteers. After reviewing the relevant features of Huntington's disease and current state of biomarker research in Huntington's disease, several approaches to, and outcomes from, biomarker discovery and evaluation are described, including proteomic profiling, targeted ELISA, multiplex inflammatory profiling and measurement of whole-brain atrophy by longitudinal magnetic resonance imaging. The thesis draws together these different approaches and summarises the contributions to both biomarker research and our understanding of the neurobiology of HD that the work has generated.

Contents

List of tables

List of figures

Abbreviations

2DE	2-dimensional electrophoresis
8OH2′dG	8-hydroxy-2-deoxyguanosine
A2M	Alpha-2-macroglobulin
Aβ	Amyloid beta
AD	Alzheimer's disease
ANOVA	Analysis of variance
Apo	Apolipoprotein
AUC	Area under the curve
B2M	Beta-2-microglobulin
BBB	Blood-brain barrier
(B)BSI	(Brain) boundary shift integral
BDI	Beck depression inventory
BDNF	Brain-derived neurotrophic factor
CAG	Cytosine-adenine-guanine
CI	Confidence interval
CNS	Central nervous system
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DME	Dimethoxyethane
DSM	Diagnostic and Statistical Manual
DTI	Diffusion tensor imaging
EDTA	Ethylene-diamine-tetra-acetic acid
EHDN	European Huntington's Disease Network
ELISA	Enzyme-linked immunosorbent assay
FAAH	Fatty acid amide hydrolase
FA	Fractional anisotropy
FAST	Fourier acquired steady state technique
FBS	Fetal bovine serum
FDR	False discovery rate
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
GC-TOF	Gas chromatography time of flight
GEE	Generalised estimating equation
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRASS	Gradient-recalled acquisition in the steady state
HD	Huntington's disease
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
ICD	International Classification of Disease
IFN	Interferon
IL	Interleukin
IS	Independence scale
KMO	Kynurenine monooxygenase
LC/MS/MS	Liquid chromatography with tandem mass spectrometry
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MALDI-ToF	Matrix-assisted laser desorption/ionisation-time of flight
MARS	Multiple affinity removal system

MIDAS	Medical Image Display and Analysis Software
MMLV	Moloney murine leukaemia virus
MR(I)	Magnetic resonance (imaging)
(m)RNA	(Messenger) ribonucleic acid
MRS	Magnetic resonance spectroscopy
MSD	Meso Scale Discovery
NCBI	National Center for Biotechnology Information
NEX	Number of excitations
NfH	Neurofilament heavy chain
NFκB	Nuclear factor κB
NHNN	National Hospital for Neurology and Neurosurgery
NHS	National Health Service
NMDA	N-methyl-D-aspartic acid
PAGE	Polyacrylamide gel electrophoresis
PBA	Problem behaviours assessment
PBS	Phosphate-buffered saline
PET	Positron-emission tomography
PM	Premanifest
(q)PCR	(Quantitative) polymerase chain reaction
ROC	Receiver operating characteristic
RT	Reverse transcription
SBA	Short behavioural assessment
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SPM	Statistical parametric map
TE	Echo time
TFC	Total functional capacity
TIV	Total intracranial volume
TNF	Tumour necrosis factor
TR	Relaxation time
UCL(H)	University College London (Hospitals)
UHDRS	Unified Huntington's disease rating scale
VBM	Voxel-based morphometry
WT	Wild-type
YAC	Yeast artificial chromosome

He shall suffer whatever events his own fate and the stern Spinners twisted into his thread of destiny when he entered the world and his mother bore him.

Homer, *Odyssey*. Book viii, 193ff.

The Three Fates, Jacob Matham (1587).

The thread of life was spun by Clotho and finally severed by Atropos; but it was Lachesis, 'the measurer', who determined its length.

Chapter I Introduction

I.1 Huntington's disease

It is spoken of by those in whose veins the seeds of the disease are known to exist, with a kind of horror, and not at all alluded to except through dire necessity, when it is mentioned as 'that disorder'.

So wrote George Huntington in 1872 in his *Medical and Surgical Reporter* paper *On chorea*, the initial report of the disease that came to bear his name (Huntington 1872). It is difficult to do justice, in describing it, to the diversity and magnitude of the effects of Huntington's disease (HD) on its sufferers, those who know they are destined to develop it, those who do not know whether they have inherited it, and their loved ones. An illustration of its horrors can be had from descriptions of HD in the usually sober scientific literature where, in addition to setting out its major features — 'neurodegenerative', 'progressive', 'inherited', 'severe', 'fatal' — more vivid descriptions go some way towards depicting the influence of HD, seen through the eyes of the researcher: 'devastating', 'dramatic', 'incurable', 'inexorable'.

Prose cannot convey the overwhelming power of HD to destroy lives and families. This introductory chapter will nonetheless set out the basic epidemiological, clinical and pathological facts of HD, before going on to review some of the reasons why there is hope for disease-slowing treatments in the near future, finally discussing the current state of biomarkers for HD, and why and how such markers may enable treatments to reach the patients and gene carriers so desperately in need of them.

I.1.1 Clinical features

I.1.1.1 General description

> *... hardly ever manifesting itself until adult or middle life, and then coming on gradually but surely, increasing by degrees, and often occupying years in its development, until the hapless sufferer is but a quivering wreck of his former self ...*
>
> (Huntington 1872)

The classical triad of clinical features of HD consists of movement disorders, psychiatric abnormalities and cognitive decline (Bates *et al.* 2002). In addition, manifestations of HD outside the central nervous system are increasingly seen as having direct relevance to the disease course (e.g. Björkqvist *et al.* 2006; Robbins *et al.* 2006). These aspects are discussed in turn below.

While, as Huntington himself described chillingly, HD generally is a disease affecting young adults, with a mean age at onset of around forty years, one of the striking features of the disease is its heterogeneity, in both age at onset and clinical phenotype, capable of beginning at any time from early childhood to old age (McCusker *et al.* 2000; Rasmussen *et al.* 2000). With exceptions, HD generally progresses slowly, especially in comparison with some of the more common neurodegenerative diseases such as Alzheimer's disease. The median interval between clinical diagnosis and death is typically given as 15 to 20 years (Roos *et al.* 1993), though it is to be hoped that the modern multidisciplinary care of HD patients is capable of sustaining good quality life for longer than this.

HD is caused by a CAG triplet repeat expansion in the IT15 gene, encoding the protein huntingtin (The Huntington's Disease Collaborative Research Group 1993). The disease is inherited in an autosomal dominant manner and the length of the expanded CAG repeat tract is a determinant of the age at onset. Repeat lengths of 39 and over are associated with full penetrance. In the expanded range, the length of the CAG repeat predicts about 50-70% of the age at clinical onset, with larger repeat lengths tending to result in earlier onset (Andrew *et al.* 1993; Duyao *et al.* 1993; Snell *et al.* 1993).

I.1.1.2 Neurological features

The most striking neurological feature of HD is the movement disorder chorea; indeed, until recently HD was known as Huntington's chorea. From the Greek κορεια (koreia), the term means literally 'a dance'. Chorea is a hyperkinetic movement disorder characterised by excessive spontaneous movements that are irregularly timed, randomly distributed and abrupt. It ranges in severity from restlessness with mild, intermittent exaggeration of gesture and expression, through fidgety movements of the hands and unstable dance-like gait, to a continuous flow of disabling and violent movements (Barbeau *et al.* 1981). Chorea is typically a relatively early motor feature and is seen in about 90% of adult-onset cases, but does not progress linearly: rather it tends to peak after a few years then gradually recede in advanced disease (Young *et al.* 1986).

Non-choreic movement disorders, including hypokinetic abnormalities such as rigidity and bradykinesia as well as dystonia (sustained involuntary contraction of muscle groups) and impairment of voluntary motor function are less prominent in very early disease but are almost ubiquitous by moderate-stage disease and, unlike chorea, tend to increase more linearly over time, to predominate by advanced disease (Thompson *et al.* 1988). While chorea is often the most obvious feature — and the most distressing to friends and relatives — non-choreic movement disorders, with their tendency to disrupt gait, balance and function, are generally more disabling than chorea, to which many patients are in fact strangely oblivious (Snowden *et al.* 1998). Indeed, symptomatic treatment of chorea with neuroleptic medications often does not produce functional improvement, because of worsening of hypokinetic features (Shoulson 1981). Rigidity and other hypokinetic features are usually prominent in juvenile-onset HD, which also carries an increased risk of seizures. About 10% of adult patients have an early bradykinetic phenotype with minimal chorea: this is known as the Westphal variant (Westphal 1883; Louis *et al.* 2000).

Oculomotor disturbance is a cardinal feature of HD: it is near-universal and frequently present as a very early, though subtle, manifestation. The key features in early disease are delayed initiation of saccadic eye movements and inability to suppress reflexive fixation on novel stimuli; later, the saccades become slowed and are accompanied by blinking or head-thrusts; in advanced disease, broken pursuits, gross saccadic slowing and gaze restriction are seen. Oculomotor abnormalities

imply a progressive degeneration of frontal-subcortical networks and, as some of the earliest changes seen in HD, are appealing as a source of potential biomarkers (Lasker *et al.* 1997).

Other neurological abnormalities include hyperreflexia, which is present in 90% of patients in early disease and implies involvement of the pyramidal tracts (Young *et al.* 1986). Dysarthria and dysphagia are very common from moderate-stage disease onwards and the latter is a major cause of morbidity and mortality. By end-stage disease, patients are almost totally disabled, fully dependent and unable to communicate. Death in Huntington's disease is most commonly caused by infection, malnutrition or suicide (Lanska *et al.* 1988).

A standardised clinical rating system for HD was first introduced in 1979 in an attempt to produce a scale capable of quantifying the main clinically-relevant disease features, both for clinical use and to standardise clinical measures between studies (Shoulson *et al.* 1979). Initially it consisted of a functional scale, to which motor and psychiatric components were added (Shoulson 1981) until formally codified as the unified Huntington's disease rating scale, with motor, behavioural, functional and cognitive components (The Huntington Study Group 1996).

The UHDRS is widely used, both clinically and in observational and interventional studies of HD. It is not without its weaknesses, however. It is virtually impossible to standardise its application completely, so it is subject to considerable inter-rater and intra-rater variability (Hogarth *et al.* 2005). More importantly, it cannot be considered to be an accurate reflection of the totality of the disease process. Certain key features, such as weight loss, are omitted altogether, while others seem to have undue prominence: of a possible 124 points on the motor scale, hyperkinetic movement disorders (dystonia and chorea) contribute 48 points while the impairment of voluntary movements contributes only 32 and bradykinesia 16, even though the latter two categories are each independently more important than chorea in contributing functional disability (Shoulson 1981; Thompson *et al.* 1988).

A key area of contention in neurological assessment in HD is the concept of 'motor onset', also known as 'phenoconversion'. In the clinical setting, and in most studies, HD is officially diagnosed as having occurred when a trained specialist examines a patient and declares "the unequivocal presence of an otherwise unexplained extrapyramidal movement disorder (e.g., chorea, dystonia,

bradykinesia, rigidity)" (The Huntington Study Group 1996; Hogarth *et al.* 2005). The UHDRS motor scale incorporates a diagnostic confidence score (Table 1) that can be scored between 0 and 4, a score of 4 being required for the formal diagnosis of HD.

Description	Score
Normal (no abnormalities)	0
Non-specific motor abnormalities (less than 50 % confidence)	1
Motor abnormalities that may be signs of HD (50 - 89 % confidence)	2
Motor abnormalities that are likely signs of HD (90 - 98 % confidence)	3
Motor abnormalities that are unequivocal signs of HD (≥ 99 % confidence)	4

Table 1 UHDRS diagnostic confidence score

Thus defined, motor onset is one of the most reliably identifiable points in the course of the disease and is certainly of great value in the clinical setting in terms of establishing a 'moment' of clinical onset and its attendant prognostic predictions. Motor onset has relatively high inter-rater agreement: in one study of 75 clinicians shown video recordings of HD subjects, the proportion of agreement (expressed as κ scores) was 0.67 for diagnostic confidence scores of 4. However, for lesser scores agreement was poor — only 0.32 (Hogarth *et al.* 2005). Since virtually all 'premanifest' gene carriers (that is, those with diagnostic confidence scores less than four) have full functional capacity, motor onset is currently the only 'hard' diagnostic criterion that could be used to power clinical trials in HD. Because the emergence of "unequivocal" motor scores takes many years on average, and because of imperfect inter-rater agreement, the sample size requirements generated by the use of motor onset are enormous: a four-year trial capable of detecting a 20% disease-slowing effect in premanifest HD would require over 3,000 participants (Paulsen *et al.* 2006).

Moreover, 'phenoconversion' defined by the onset of specific motor features overlooks the crucial fact that for many subjects, motor abnormalities are not the first or dominant features of the illness. While 90% of adult-onset patients do develop chorea at some point during the illness, many of the most severely affected (juvenile and Westphal-variant cases) never do; meanwhile, many patients experience many years of cognitive or behavioural disturbances that, in retrospect, can be seen to have been the earliest manifestations of HD. In a study of 960 patients diagnosed as having

undergone 'motor onset', motor abnormalities were retrospectively felt by the clinician to have been the earliest disease symptom or sign in only 56%. Psychiatric, cognitive and multifactorial presentations were almost as common and there is no reason to suppose that subjects with such presentations lacked neuropathology of a degree comparable with that seen in patients with early motor signs.

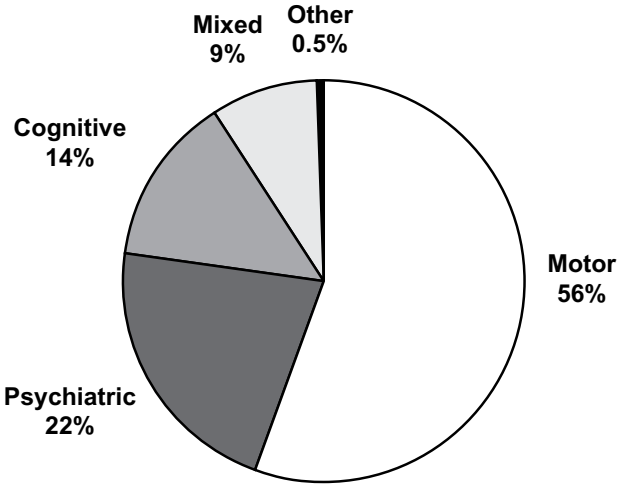

Figure 1 Initial presenting feature of HD, determined retrospectively by clinician in 960 patients with 'manifest' disease

(Adapted from Marder et al. *2000.)*

Thus, while 'motor onset' has value to the physician and to the patient, as a means of determining mutually that the stage of progressive functional decline has begun, as a surrogate measure of neuropathological equivalence, it leaves much to be desired. Sadly, there is no unified measure of the fundamental severity of HD that can be used to compare all HD gene carriers — both with and without motor signs — directly. This highlights the need for measures that can be applied agnostically across all gene carriers to give an objective measure, related to neuropathology, and have clinically relevant predictive value: a central aim of the search for biomarkers and of this thesis.

I.1.1.3 Psychiatric features

> *The tendency to insanity, and sometimes that form of insanity which leads to suicide, is marked ... As the disease progresses the mind becomes more or less impaired, in many amounting to insanity, while in others mind and body both gradually fail until death relieves them of their sufferings.*

(Huntington 1872)

Psychiatric manifestations of Huntington's disease constitute some of the most disabling features, yet are sometimes among the more treatable, at least symptomatically. As alluded to by Huntington, the emergence of psychiatric disturbances at some point during the illness is near-universal but varies greatly in severity (Paulsen *et al.* 2001).

Many patients become depressed and, while the social and lifestyle effects of living with the effects of an incurable degenerative disease in oneself and one's family are far from negligible, there is consensus that depression in HD has its basis in neurodegeneration (Mindham *et al.* 1985) The cross-sectional prevalence of depression in HD high, around 50% (compared with 4% in the general population), and the onset of depression frequently predates the diagnosis of manifest HD by several years (Folstein *et al.* 1983; Folstein *et al.* 1987; Pflanz *et al.* 1991). As expected with such a high prevalence of depression, suicide, attempted suicide and self-harm are common: in one study suicide as a cause of death (likely an underestimate of all cases) was four times more common in HD patients than in the population (Farrer 1986).

Anxiety disorder is a frequent accompaniment of depression in HD and can also occur in isolation and be disabling. Irritability and apathy are well-recognised clinically and by family members and carers, and can be highly challenging behaviours for all concerned (Paulsen *et al.* 2001). Other psychiatric disorders such as psychotic illness and mania appear to be more common in HD than in the general population (Pflanz *et al.* 1991). Sexual disinhibition and inappropriate behaviour are probably less common than generally held, because of the excessive tendency of such behaviours to be reported. The commonest sexual dysfunctions in HD are in fact hypoactive sexual desire and inhibited orgasm (Fedoroff *et al.* 1994).

The optimal assessment of psychiatric features in HD is a matter of debate. Psychiatric phenomena —disturbances of thought and mind — are by definition impossible to assess objectively, so behavioural measures, based on reports and outward manifestations of psychiatric disturbance, must be relied upon. Traditional formal clinical criteria for the diagnosis of psychiatric abnormalities, such as the International Classification of Diseases (ICD) and Diagnostic and Statistical Manual (DSM), specifically preclude diagnosis of individual syndromes in the presence of an organic brain disease such as HD (World Health Organization 1992; American Psychiatric Association 1994). Generic scales such as the Beck Depression Inventory (Beck *et al.* 1996) and Hospital Anxiety and Depression Scale (Zigmond *et al.* 1983) lack questions about important behavioural manifestations of HD, such as irritability, or ask questions that may be confounded by non-behavioural features, as in the BDI's question about weight loss, that may render the results unreliable.

The UHDRS behavioural assessment, used in the REGISTRY study, is a semi-structured interview that seeks to quantify the severity and frequency of symptoms in each of the following domains: depressed mood; low self-esteem/guilt; anxiety; suicidal thoughts; disruptive or aggressive behaviour; irritable behaviour; perseverative/obsessional thinking; compulsive behaviour; delusions; hallucinations; apathy; and behavioural milestones (The Huntington Study Group 1996; Euro-HD Network REGISTRY Steering Committee 2003-8). However, the UHDRS behavioural assessment, while widely used, is also widely criticised for lacking precise operational criteria (vague terms like "seldom", "sometimes" or "frequently", for example) and for a poorly capturing the spectrum of behavioural impairments in HD. For instance, perseverative/obsessional behaviour and compulsive behaviour are often impossible to dissociate in HD, in which the persistent, recurrent thoughts and behaviours are better described as 'perseverative' rather than in the terminology of obsessive-compulsive disorder. The Problem Behaviours Assessment (Craufurd *et al.*) stemmed from an attempt to re-evaluate the behavioural features of HD based on theoretical features of interest from publications of disorders of the frontal lobes, symptoms mentioned during clinical consultations and features from the literature in HD. The result was a forty-point questionnaire that, while thorough and evidence-based, was also redundant and too long in the administration for use in a multimodal assessment study such as this.

The Behavioural Phenotype Working Group of the Euro-HD network has as a central aim the development and improvement of available behavioural assessment tools. Working collaboratively, the working group has developed the Short Behavioural Assessment, a questionnaire intended to replace the UHDRS behavioural assessment as a quickly-administered assessment encompassing the main domains of behavioural dysfunction in HD in a comprehensive yet parsimonious tool that can be administered in 15-30 minutes. It combines features of both the UHDRS behavioural assessment with refinements derived from the longer PBA. It takes the form of a structured interview and is accompanied by consensus guidelines for its administration and scoring, as well as a formalised training programme to improve standardisation.

I.1.1.4 Cognitive features

Progressive multi-domain cognitive decline, amounting to a form of dementia, is a universal feature of HD, frequently emerging early in the clinical course or even several years before the emergence of neurological signs (Craufurd *et al.* 2002; Johnson *et al.* 2007; Solomon *et al.* 2007). In keeping with the distribution of pathology (and, by retrograde extension, neuronal dysfunction), the cognitive phenotype of HD is generally described as a frontal-subcortical syndrome, with abnormal activity in the frontal cortex and basal ganglia likely contributing throughout the illness (Brandt *et al.* 1986). Cognitive decline is a major source of concern for patients and carers, and a leading predictor of functional impairment (Marder *et al.* 2000).

Executive function impairment is the most striking cognitive feature of HD. Difficulty with multi-tasking, impaired concentration, impulsivity, excessively rigid thinking and psychomotor slowing are features of the dysexecutive syndrome, the severity of which correlates with striatal and insular atrophy (Peinemann *et al.* 2005). Unlike disorders selectively affecting frontal cortical function, HD is also characterised by psychomotor slowing, and in contrast to other causes of dementia, language, visuospatial and visuoperceptual function tend to be relatively preserved (Craufurd *et al.* 2002). Apparent deficits in memory are generally due to problems of organisation and attention rather than an amnestic syndrome *per se* (Lundervold *et al.* 1994).

The cognitive phenotype of the longitudinal imaging study cohort described in this work has been characterised in detail in another thesis, so is not reported in detail here (Henley 2008).

I.1.1.5 Systemic features

The clinical features of HD are seldom confined to the central nervous system. Huntingtin is expressed ubiquitously and it is therefore perhaps unsurprising that the mutant protein should produce a phenotype outside the CNS as well as within it. Prominent weight loss, sometimes amounting to cachexia, is an invariable feature of advanced HD but is also frequently seen earlier in the disease course (Sanberg *et al.* 1981; Farrer *et al.* 1985; Morales *et al.* 1989; Aziz *et al.* 2008). Low weight (i.e. low body mass index) is associated with more rapid progression of HD (Myers *et al.* 1991). The additional calorific burden of the hyperkinetic movement disorder appears inadequate to explain the weight loss of HD, which may relate to an underlying energy utilisation deficit (Mochel 2007). Weight loss is often accompanied by muscle wasting (Sanberg *et al.* 1981), which is probably at least partially due to direct effects of the mutation on muscle cells (see section I.1.4.4). HD patients appear to have decreased beat-to-beat variability of the heart rate (Sharma *et al.* 1999), suggesting cardiovascular involvement in HD. The endocrine system represents a nexus between the CNS and peripheral tissues and endocrine disturbances, not entirely explicable by hypothalamic dysfunction, are a feature of HD: the prevalence of diabetes mellitus appears to be higher than in the general population (Farrer 1985) and testicular size and hormonal profile are altered (Markianos *et al.* 2005; Van Raamsdonk *et al.* 2007).

Many of these peripheral features contribute to HD patients' morbidity and mortality directly, and may also contribute indirectly to CNS dysfunction. The notion of exploiting these peripheral disease-related changes as a possible source of biomarkers is discussed in sections I.1.4.4 and I.2.

I.1.2 Genetics of Huntington's disease

> *When either or both the parents have shown manifestations of the disease ... one or more of the offspring almost invariably suffer from the disease, if they live to adult age. But if by any chance these children go through life without it, the thread is broken and the grandchildren and great-grandchildren of the original shakers may rest assured that they are free from the disease.*
>
> (Huntington 1872)

That IT15 ('interesting transcript 15'), the gene encoding the huntingtin protein, is the cause of HD, was discovered in 1993 after a major and unprecedented international collaborative effort (The Huntington's Disease Collaborative Research Group 1993). The causative mutation is an expansion of a CAG triplet repeat tract in exon one, from the normal range of up to 29 repeats into the disease range of over 39 repeats. In the disease range, the mutation is essentially fully penetrant, with 100% of HD patients expected to develop disease manifestation unless they die prematurely from another cause. The expansion is now seen as fully sensitive and specific for HD, such that HD-like disorders in patients lacking it are referred to as HD phenocopies and alternative causes for their condition are sought, and no condition other than HD is known to be caused by the mutation.

As mentioned above, the length of the CAG repeat tract has a significant impact on the disease course, specifically on the likely age at which disease signs will emerge. The correlation was discovered shortly after the gene itself and repeat length appears to account for between 50 and 70% of the variation in age at onset (Andrew *et al.* 1993; Duyao *et al.* 1993; Snell *et al.* 1993). These findings have been confirmed in many large cohorts and the relationship between CAG repeat length and onset was recently comprehensively examined by Langbehn and colleagues. As shown in Figure 2, repeat lengths of 40 and above are associated with over 90% lifetime probability of motor onset, assuming a life expectancy of 80 years. Each successive CAG repeat shifts the cumulative onset probability curve to the left, indicating earlier likely ages at onset, but the effect is not linear, so that the age difference from 40 to 42 is greater than that from 54 to 56. Conversely, the curves are steeper for higher repeats, indicating a tighter range of likely onset ages. So, for an individual with 40 repeats, the probability of onset increases from 10% to 90% over a period of 27 years; for someone with 56 repeats, that probability range spans just 15 years (Langbehn *et al.* 2004).

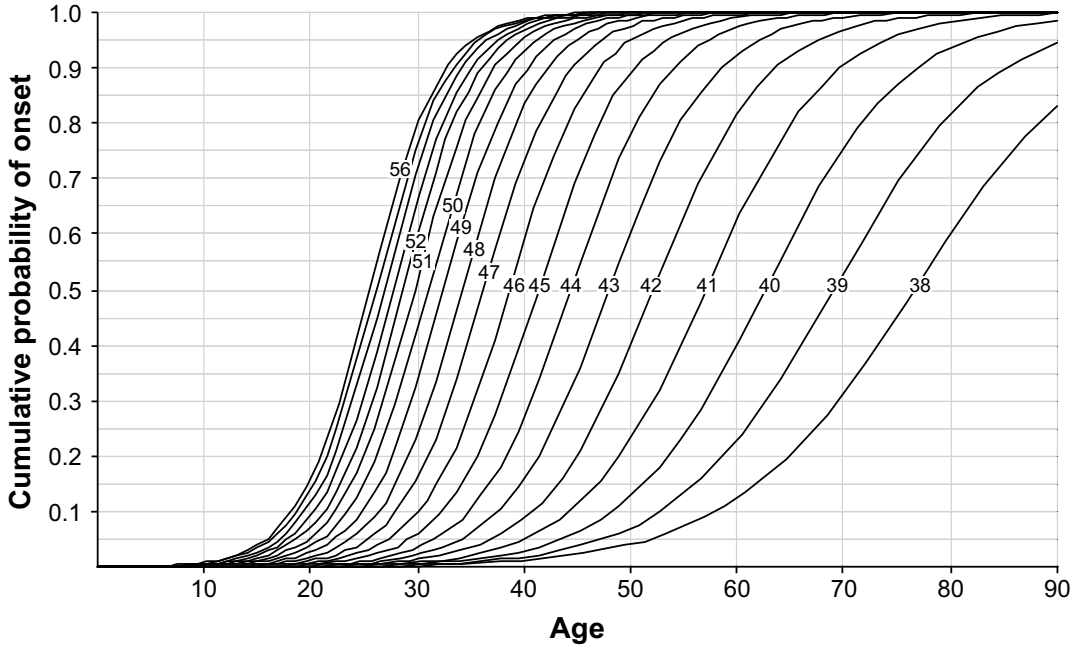

Figure 2 Cumulative probability of motor onset for a given age, by CAG repeat length

(Reproduced from Langbehn et al. *2004 by permission of Wiley, Inc.)*

Figure 3, based on data from the same study, shows the relative frequencies of each pathological repeat length. The most common length is 43 triplets, and the majority of alleles are tightly distributed with repeats between 42 and 45, towards the lower end of the observed sizes, in the range where the range of likely ages of onset is very wide. The study also gave rise to the now widely-used formula for the calculation of an individual's probability of motor onset over a chosen interval, given that he or she is currently without motor signs. This calculation, which is of great value in assessing the extent to which a candidate biomarker is associated with known genetic predictors of disease, is discussed in detail in chapter II.11.

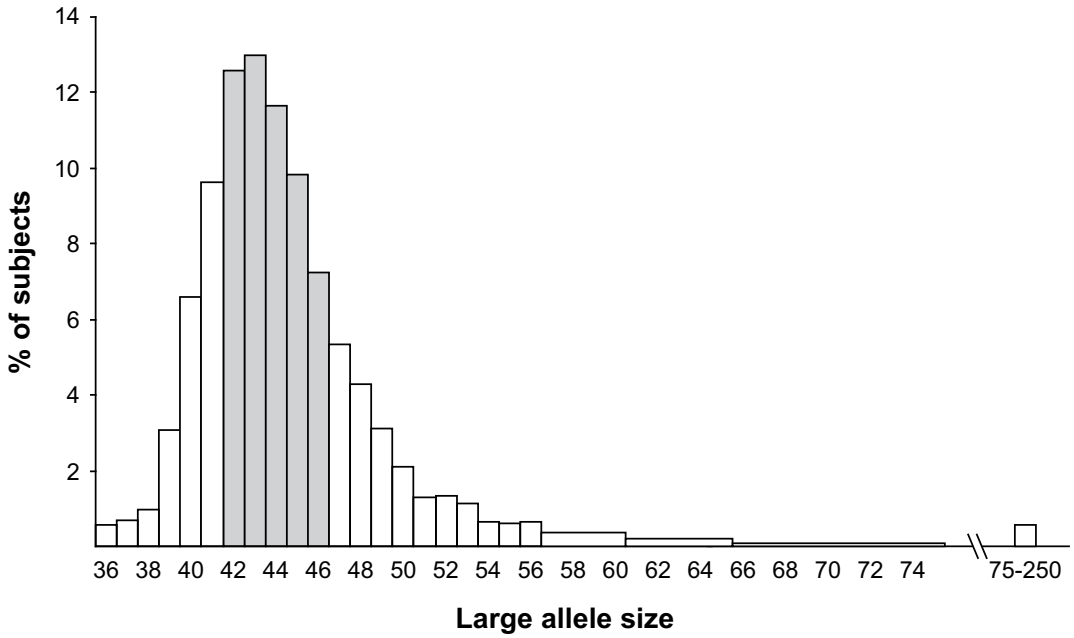

Figure 3 Frequency of expanded alleles in 3452 subjects from 40 centres

(Adapted from Langbehn et al. *2004.) Grey shaded bars represent those allele sizes falling in the 25th to 75th percentiles.*

Almost all cases of juvenile-onset HD occur patients with large CAG repeats, typically over 50, and juvenile patients usually have a rapidly progressive akinetic-rigid presentation (Telenius *et al.* 1993; Sánchez *et al.* 1996). Juvenile HD apart, CAG repeat length is a poor predictor of phenotypic characteristics other than age at onset. Sources of the great clinical heterogeneity of HD, such as the balance of motor and non-motor symptoms, cannot be predicted based on CAG repeat length (MacMillan *et al.* 1993). Whether CAG repeat length influences rate of progression is a controversial topic, subject as it is to the analytical hazards of the inextricably-linked interrelationships between repeat length, onset age and disease duration discussed in chapters II.3.1.3 and II.11.10. It is clear that subjects whose disease manifests at a younger age progress more quickly, but proving an additional effect of CAG repeat length on rate of progression that survives statistical correction for age at onset *per se* is problematic. Early studies failed to find an effect at all (e.g. Kieburtz *et al.* 1994) while others found a trend that did not survive correction for the effect of age at onset (Brandt *et al.* 1996). However, more recent studies using larger subject numbers and standardised clinical measures do suggest an effect of CAG repeat length on clinical

and functional progression independent of age-at-onset (Mahant *et al.* 2003; Ravina *et al.* 2008). Unsurprisingly, there appears to be an effect of CAG repeat length on the severity of pathology, after correction for age at onset, a finding that led to the 'disease burden score' calculation discussed in chapter II.11.10 (Penney *et al.* 1997). Several proposed biomarkers of progression across assessment modalities have been shown to correlate independently with CAG repeat length; section I.2 below mentions these in turn.

That HD is caused by a triplet repeat expansion is the explanation for the phenomenon of anticipation, which describes the tendency for an inherited disease to emerge earlier, or with a more severe phenotype, with each successive generation. Anticipation in HD is caused by a combination of the causative relationship of CAG repeat length with age at onset, and the tendency for the length pathogenic expansions to increase, on average, with each generation (Andrew *et al.* 1993; Duyao *et al.* 1993; The Huntington's Disease Collaborative Research Group 1993). Interestingly, intergenerational repeat-length expansions are sexually dimorphic: while small changes of up to seven repeats are seen in both male and female transmissions, with an overall mean change of zero, transmissions from male expansion carriers are disproportionately prone to large changes in repeat length, producing a mean intergenerational increase of +4 repeats from paternal inheritance. Moreover, the larger the parental repeat length, the more likely is a large expansion on transmission. This explains why the majority of juvenile HD cases are inherited from an affected father (Ranen *et al.* 1995). The mechanisms underlying the male-specific expansion of HD alleles are poorly understood. Clearly males are differentially susceptible to unfavourable germ-line mutations and it has been suggested that the CAG repeat tract allowing 'slippage' of the DNA replication apparatus, combined with the greater number of cell divisions in spermatogenesis compared with oogenesis, may underlie the phenomenon. However, other triplet-repeat diseases such as fragile X syndrome and spinocerebellar ataxia type 7 tend to expand through the maternal line, so this is clearly an incomplete explanation (Pearson 2003).

Whereas repeat lengths of 39 and above may be considered fully penetrant, and those under 29 repeats are normal, lengths between these values are a cause for diagnostic uncertainty. The lower end of the range — 29 to 35 repeats — is usually referred to as the 'intermediate allele' range. Between 1% and 4% of the general population carries alleles in this range. While they do not cause

a clinical phenotype themselves — reported exceptions remain controversial (Kenney *et al.* 2007; Semaka *et al.* 2008) — there is concern that they may expand into the pathogenic range in future generations (Semaka *et al.* 2006). Repeat lengths in the range of 36 to 39 are referred to as 'reduced penetrance' alleles and may or may not produce a clinical HD syndrome during an individual's natural life (Langbehn *et al.* 2004). As shown in Figure 2, the distinction between these and 'fully penetrant' alleles is purely quantitative and, unlike alleles of fewer than 36 repeats, it is likely that they would eventually produce a clinical phenotype if the subject were to live sufficiently long.

The co-existence of anticipation and intermediate and reduced-penetrance alleles is the likely explanation for the continued prevalence of HD, despite its apparently self-terminating tendency. Were it not for the ability of normal alleles to expand into the intermediate range, and for subjects lacking a clinical phenotype thereby to pass on an expanded allele to their offspring, each HD pedigree would tend to cease with a case of juvenile HD resulting in no offspring. There appears to be an inexhaustible supply of fresh mutations and new pedigrees with each passing generation.

I.1.3 Epidemiology

> *... the disease exists, so far as I know, almost exclusively on the east end of Long Island ... confined to certain and fortunately a few families.*
>
> (Huntington 1872)

Sadly, on the epidemiology of HD, George Huntington was mistaken. It is now known to be the third most common inherited neurological disorder in the United Kingdom, after type I neurofibromatosis and Charcot-Marie-Tooth disease (MacMillan *et al.* 1991). Worldwide, HD has been found in every population where it has been sought, and tends to have a prevalence (for manifest disease) of 2.5-10 per 100,000, with the consensus from the most reliable studies settling around 4 per 100,000 (Bates *et al.* 2002). Pockets of low (Japan, Finland) and high (Tasmania, Venezuela) prevalence are known to exist (Narabayashi 1973; Young *et al.* 1986; Palo *et al.* 1987; Pridmore 1990). Prevalence in the United Kingdom lies in the typical range for Western Europe (Bates *et al.* 2002), meaning that there are between 2400 and 6000 individuals with HD in the UK.

Half of those with an affected parent will inherit an expanded allele, but the disease generally remains asymptomatic for many years, only a minority of those at risk undergo testing, and some mutation carriers are unaware of their at-risk status. There are therefore many more individuals at risk, or gene-positive but prior to clinical diagnosis, than there are manifest HD patients. Estimates of the prevalence of the mutation (summarised in Figure 4) suggest that for every ten people with manifest disease, there are forty at-risk individuals, of whom fifteen carry the mutation (Walker *et al.* 1981; Conneally 1984). Overall there are around 12,000 at-risk individuals (including gene-negatives, premanifest gene carriers and manifest HD patients) in the UK.

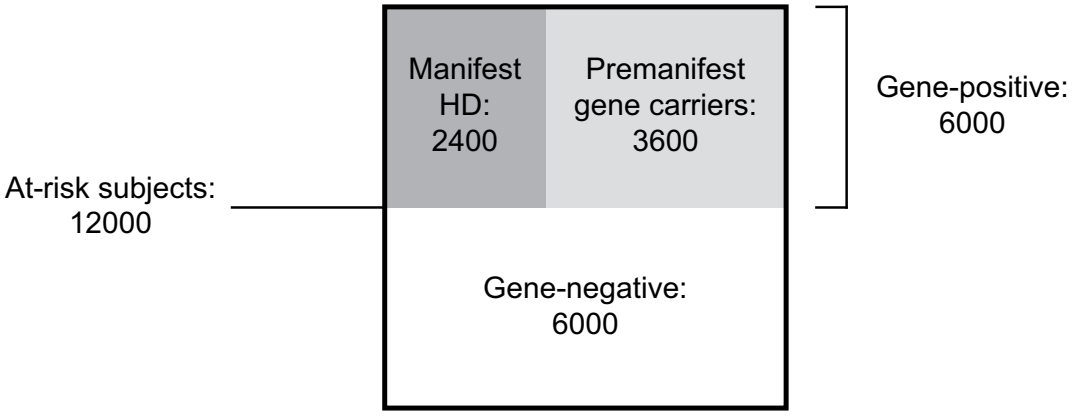

Figure 4 Prevalence estimates for the United Kingdom for subjects at risk of HD, gene negative individuals, premanifest carriers and manifest HD patients

Estimates are based on the lower end of the range for UK prevalence of 4 per 100,000 and a low-end population estimate of 60 million.

Large though these prevalence figures are, they cannot, of course, do justice to the collateral damage that HD continues to wreak: the psychosocial cost to those at risk of inheriting it of witnessing, often during their childhood, the slow, unremitting decline and death of at least one relative; the effect of the knowledge that they are at risk of the same condition; and the impact of the disease over many years on those left to care for the patients. Overall it is clear that HD casts a long shadow over very many families.

I.1.4 Pathobiology of Huntington's disease

> *But even if we take it for granted that we have discovered the* sedes morbi *we are still left in ignorance in regard to the nature of the derangement. And here we must leave the interesting subject of the pathology of this disease, and trust that the science, which has accomplished such wonders, through the never-tiring devotion of its votaries, may yet 'overturn and overturn, and overturn it,' until it is laid open to the light of day.*
>
> (Huntington 1872)

While knowledge that HD is caused by a single, fully penetrant mutation has led in the past fifteen years to a dramatic illumination of its molecular and cellular pathobiology, it cannot yet be considered "laid open to the light of day" and much remains unknown about the processes that lie between the mutation and its ultimate manifestations. A full review of the pathobiology of HD is beyond the scope of this thesis; after summarising the main features, this section will concentrate on aspects of pathology relevant to the search for HD biomarkers and the specific work presented in later chapters.

I.1.4.1 Macroscopic pathology

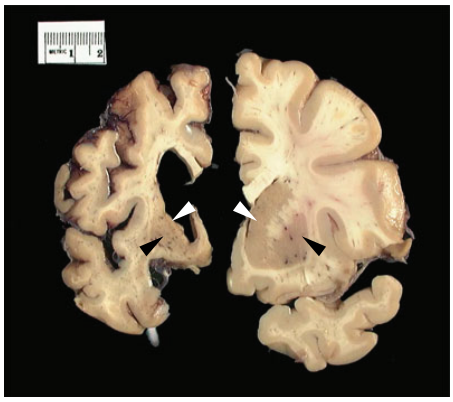

Figure 5 Comparison of coronal slices from fixed cerebral hemispheres of an HD patient and matched control subject

HD patient on left, control subject on right. White arrowheads, caudate nuclei; black arrowheads, putamina. Image courtesy of Harvard Brain Tissue Resource Center.

Macroscopically, HD brains weigh 10-20% less overall than matched control brains and it is obvious on inspection of gross specimens from advanced cases (Figure 5) that brain atrophy in HD is widespread and marked. The combined volume of both striata in an adult control seldom exceeds 30mL (Aylward *et al.* 2004), less than 3% of the total brain volume of a typical adult (around 1200mL). Thus, 70-90% of atrophy in HD must be extrastriatal. This consideration, with its implications for the ease and accuracy of measurement of whole-brain volume loss, as well as the likely clinical effects of that degree of extrastriatal atrophy, argues for the study of whole-brain atrophy, as discussed in Chapter VII, as a biomarker of HD progression. Despite these impressive whole-brain changes, it is clear that the striatum is differentially affected by atrophy. The caudate and putamen are virtually abolished by end-stage HD (Figure 5) and are the earliest structures to lose volume (Forno *et al.* 1979). Many of the early clinical abnormalities of HD, such as chorea, dystonia and the typical 'subcortical' dysexecutive cognitive syndrome, are explicable by striatal atrophy but would be expected to be worsened by cell death in other brain regions, especially those having connections with the striatum (Albin *et al.* 1989). Crucially for the discussions of Chapter VI and Chapter VII, measurement of atrophy using volumetric MRI can provide a marker of neuronal loss (Fox *et al.* 1996).

I.1.4.2 Microscopic pathology

Echoing the macroscopic picture, the neostriatum is most severely affected on the microscopic level in HD, with neuronal loss and astrogliosis (Figure 6). The widely-used pathological grading system of Vonsattel and colleagues is based on the severity of pathology in the striatum and divides subjects into five grades (0 to 4) based on the extent of macroscopic atrophy, neuronal death and astrocytosis in the caudate, putamen, globus pallidus and nucleus accumbens (Vonsattel *et al.* 1985).

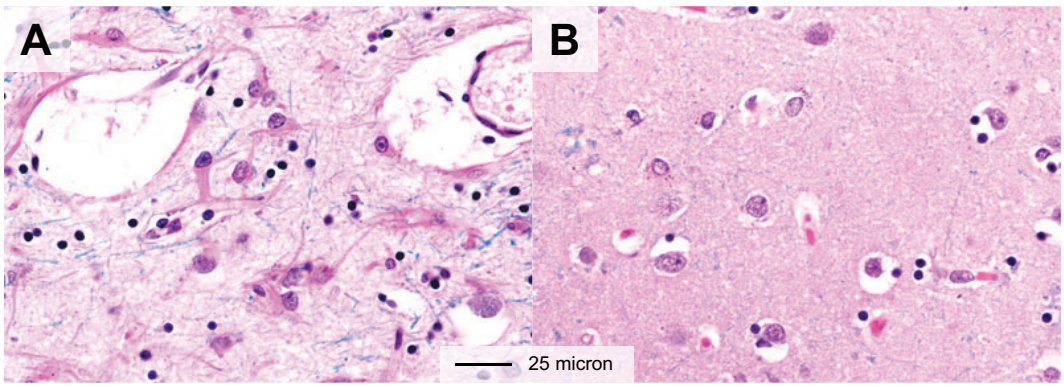

Figure 6 Microscopic HD pathology in the striatum

A. *HD striatum showing neuronal loss and astrocytosis (dark coloured processes).* ***B***. *Striatum from age-matched control subject showing healthy neurons and a uniform background without astrocytosis. Haematoxylin and eosin stain. Image courtesy of Harvard Brain Tissue Resource Center.*

Within the striatum there is differential involvement of cell populations. Worst affected are the inhibitory GABAergic medium spiny neurons, which project to the globus pallidus and substantia nigra (Reiner *et al.* 1988). Why these cells should be selectively involved remains unclear but differential influences such as growth factors, excitotoxicity, neurotransmitter gene expression, somatic CAG repeat expansion and afferent anatomical connections have been suggested to contribute (Sieradzan *et al.* 2001; Gonitel *et al.* 2008)

Beyond the striatum, most brain regions show pathological involvement to some extent. The cerebral cortex displays neuronal loss, affecting primarily layers III, IV and VI (Tellez-Nagell *et al.* 1973). As has since been confirmed by MRI cortical thickness measurement (Rosas *et al.* 2002; Rosas *et al.* 2008), cortical atrophy is relatively uniform by end-stage, with relative sparing of the medial temporal lobes, and correlates with both CAG repeat length and the degree of striatal atrophy (Halliday *et al.* 1998). The frontal and prefrontal cortex, with its connections to the striatum, has long been known to be affected in HD and cortical cell loss has sometimes been considered secondary to striatal atrophy (Cudkowicz *et al.* 1990). However, recent work suggests that the pattern of regional neuropathology differs between clinical phenotypes, with predominantly motor cases having more cell loss in the motor cortex and predominantly psychiatric cases having greater loss in the cingulates and secondary visual cortex (Thu *et al.* 2008). Such region-specificity in cortical

pathology between different presentations argues for selective cortical pathology as a primary and clinically-relevant determinant of phenotype, a possibility investigated in Chapter VII. Other brain regions with potentially important functional links to phenotypic characteristics of HD have been found to be involved, including the cerebellum (Jeste *et al.* 1984), hypothalamus (Kremer *et al.* 1991) and subcortical white matter (Halliday *et al.* 1998). Importantly, CAG repeat length is not constant throughout the brain, but displays regional mosaicism, and those areas where neuropathology is most pronounced, including the striatum and cerebral cortex, tend to be those where repeat length variability is greatest (Telenius *et al.* 1994).

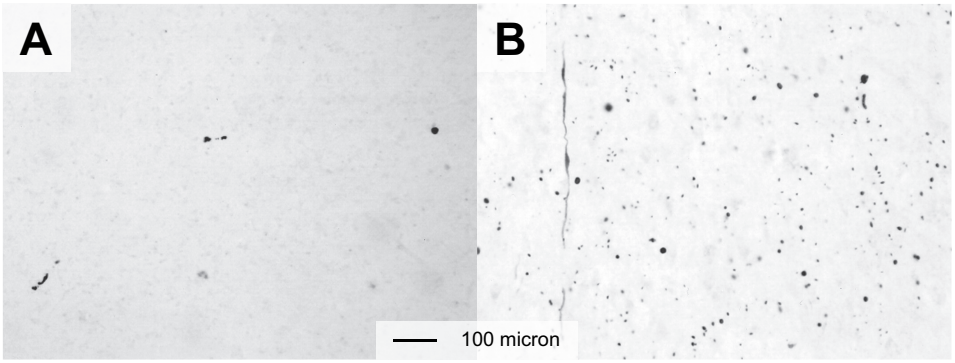

Figure 7 Neuropil aggregates in premanifest HD

*Images taken from (**A**) striatum and (**B**) cortex of a premanifest HD mutation carrier. Immunohistochemical staining with EM48, an antibody to the N-terminal region of huntingtin. (Reproduced from Gutekunst* et al. *1999 by permission of The Society for Neuroscience.)*

Immunohistochemical studies have shown that the huntingtin protein is widely expressed in neurons throughout the normal brain (Gutekunst *et al.* 1995). Areas of most severe pathology generally have high levels of normal huntingtin expression, though the converse is not true: huntingtin expression does not predict neuropathology (Ferrante *et al.* 1997). Mutant huntingtin forms ubiquitinated intracellular aggregates (Figure 7), to which other proteins are recruited. These aggregates can be in the neuropil, cytoplasmic, perikaryal or intranuclear. Curiously, while they are abundant in the cerebral cortex and elsewhere, aggregates are relatively sparse in the striatum (Gutekunst *et al.* 1999). Consequently there is much debate over whether mutant huntingtin aggregates are harmful, protective or benign. On the one hand, injecting aggregates into cells recapitulates the toxic effects of expressing mutant huntingtin (Yang *et al.* 2002) while on the other,

aggregates sequester mTOR, activating the autophagy pathway that may enhance the removal of toxic polyglutamine species (Ravikumar *et al.* 2004).

Neuronal tissues have understandably been a focus of study in HD. However, key contributions of glial cells are increasingly being recognised. Chapter III and Chapter IV discuss a possible role for inflammation in the pathogenesis of HD and suggest a prominent role for microglial cells, the CNS equivalent of peripheral monocytes and macrophages. Microglial activation has been demonstrated in HD brain (Figure 8) and microglia have been shown to come into direct contact with neuronal cells containing huntingtin aggregates (Sapp *et al.* 2001).

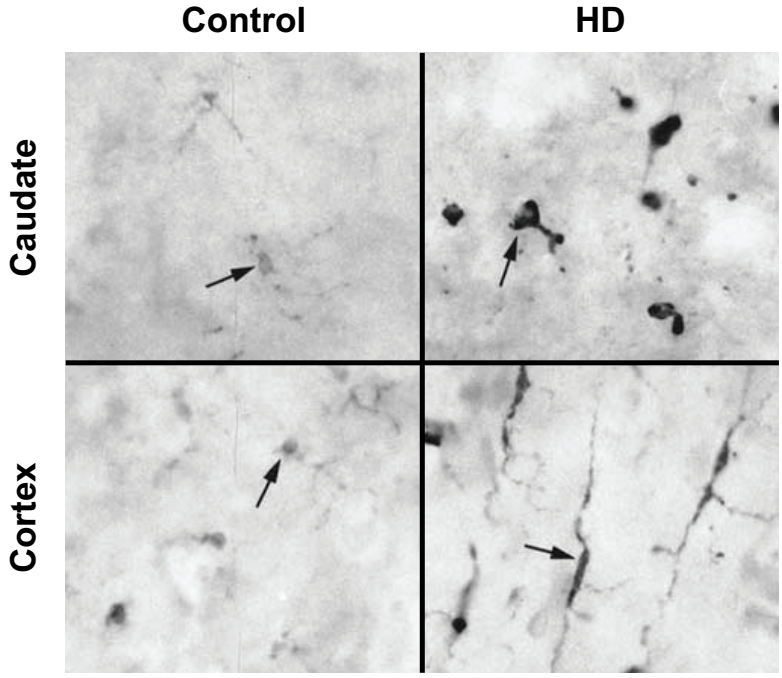

Figure 8 Microglial activation in HD brain demonstrated using Tβ4 immunostaining

Microglia (arrowheads) are only faintly labelled in control brain but prominent activation is seen in HD in both caudate and cortex, where the activated microglia adopt differing morphologies. (Reproduced from Sapp et al. *2001 by permission of Wolters Kluwer / LWW.)*

I.1.4.3 Molecular and cellular pathology

Though pathogenic roles have been proposed for mutant huntingtin DNA and RNA (e.g. Galvao *et al.* 2001), the consensus view is that the mutant huntingtin protein is the toxic species that causes HD. Huntingtin is a 348kDa protein, expressed widely in tissues and in most intracellular

compartments. After fifteen years of intensive study, though its normal function is incompletely understood, it is thought to have diverse functions in vesicle transport, cytoskeletal anchoring, neuronal transport, postsynaptic signalling, cytoprotection and transcriptional regulation (reviewed in Imarisio *et al.* 2008). While embryonic knockout of huntingtin is lethal in mice, hemizygosity does not produce a disease phenotype in humans (Landles *et al.* 2004), and HD homozygotes do not possess a more severe phenotype than heterozygotes (Wexler *et al.* 1987); most researchers have therefore focused on the hypothesis that HD is caused by a toxic gain of function of mutant huntingtin.

The presence of the CAG repeat sequence in the IT15 gene encodes a polyglutamine (polyQ) tract in the huntingtin protein. An expanded polyQ domain in the N-terminal portion of the protein induces conformation changes in the protein that result in inclusion formation. However, the n-terminal fragment is sufficient to cause selective HD-like pathology, indicating that interactions of the polyglutamine tract prior to aggregate formation may be central to HD pathogenesis (Li *et al.* 2000).

That mutant huntingtin can cause death of neurons is clear, but the relationship between clinical phenotype and neuronal death is not straightforward. In one study, five patients with end-stage clinical HD had grade 0 pathology post-mortem (Myers *et al.* 1988). In the R6/2 HD mouse model, there is minimal striatal cell death despite a pronounced, progressive neurological phenotype (Carter *et al.* 1999). It is therefore clear that a long period of neuronal dysfunction precedes cell death and is sufficient to produce the disease phenotype. This has clear implications for the search for both disease-modifying therapies and biomarkers of progression. Treatments that prevent cell death, or biomarkers that signal cell death, are unlikely to be as useful as treatments that slow or reverse dysfunction, and biomarkers that can detect it. Finally, a conditional mouse model of HD has demonstrated that if the pathogenic processes arising from the expression of mutant huntingtin can be halted, both pathological and clinical progression are not only halted but reversed, suggesting that disease-modifying treatments have the potential to bring about clinical improvement well into the symptomatic phase of the disease (Yamamoto *et al.* 2000).

The intracellular interactions and effects of mutant huntingtin are diverse, and numerous complementary pathways likely contribute synergistically to its detrimental effects (reviewed in

Landles *et al.* 2004). As summarised in Figure 9, the main mechanisms of dysfunction are felt to be gene transcriptional dysregulation, disordered protein folding, and deficient protein degradation.

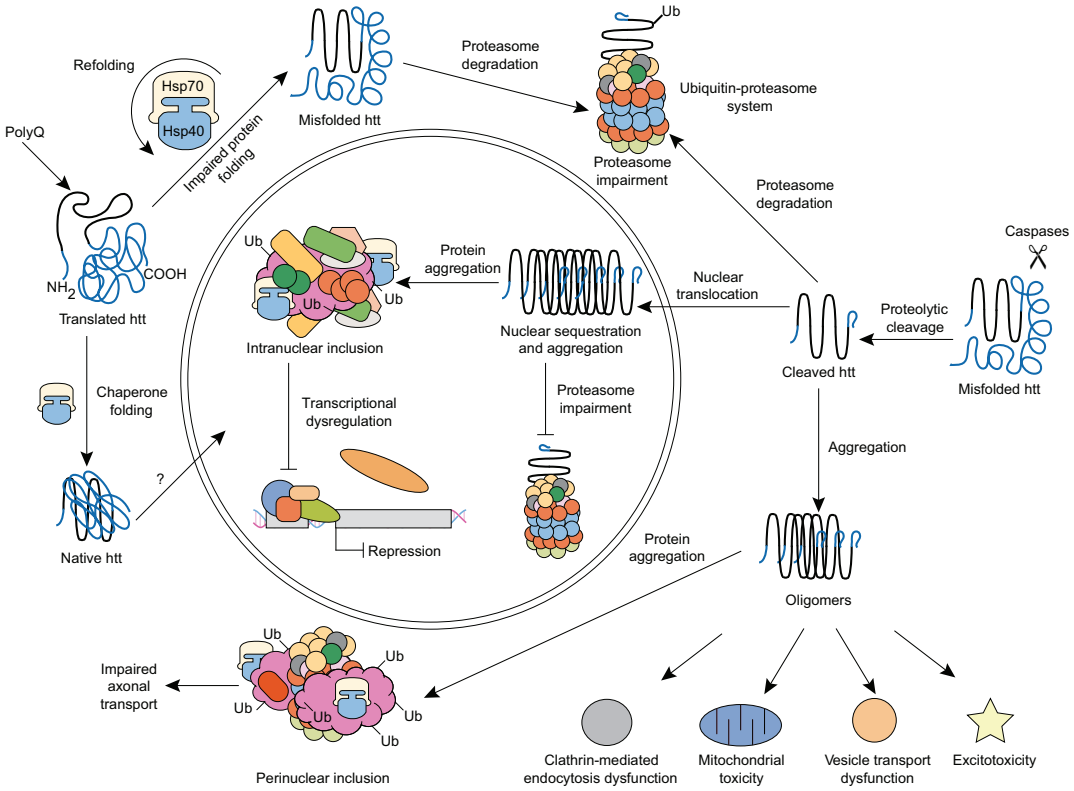

Figure 9 Summary of the cellular pathogenesis of HD

htt, huntingtin; Ub, ubiquitin. (Adapted from Landles et al. *2004 by permission of Nature publishing group; modifications after Imarisio* et al. *2008.)*

Huntingtin, in both wild-type and mutant forms, not only interacts with a wide variety of transcription factors with key roles in neuronal function and survival (reviewed in Sugars *et al.* 2003) but also binds directly to DNA, altering the function of gene promoters in a polyglutamine-dependent manner (Benn *et al.* 2008). Among the specific pathways deranged are the cAMP-responsive element (CRE) and specificity protein 1 (SP1) pathways, critical to neuronal survival (Sugars *et al.* 2003), and the neuron-restrictive silencer element (NRSE) system that enables the cortex to provide trophic support to the striatum via the axonal delivery of brain-derived neurotrophic factor (BDNF) (Zuccato *et al.* 2003). Critically to the findings discussed in Chapter IV, mutant huntingtin has been shown to activate the IκB kinase complex, allowing nuclear entry of

NFκB — an important transcriptional activator of inflammatory genes — and enhancing the toxicity of mutant huntingtin (Khoshnan *et al.* 2004). More generally, the function of essentially any cellular pathway may be deranged directly or indirectly by transcriptional dysregulation and microarray studies have shown that gene expression in HD is widely deranged (Hodges *et al.* 2006).

The ubiquitin-proteosome system is responsible for degrading unwanted small cellular proteins into amino acids. Mutant huntingtin aggregates are ubiquitinated, indicating that they are targeted for removal by the proteosome but cannot be degraded by it (Ciechanover *et al.* 2003). The proteosome cannot process peptides containing polyglutamine tracts (Venkatraman *et al.* 2004), and the UPS is almost completely inhibited in the presence of polyQ-containing mutant huntingtin fragments (Bence *et al.* 2001). The loss of function of this key system could cause widespread dysfunction of other cellular processes.

Chaperone proteins are responsible for ensuring the correct folding of cellular proteins. As well as the polyglutamine tract conferring an abnormal structure on huntingtin *per se*, aggregates have been shown to sequester key chaperone proteins (Hsp70 and Hsp40) whose expression might otherwise be beneficial for cell survival (Bonini 2002; Hay *et al.* 2004).

A number of other interactions of huntingtin highlight potential pathogenic pathways and suggest possible therapeutic approaches. Huntingtin is cleaved by caspases, specifically caspase 3 and 6, and prevention of caspase-6 cleavage prevents neurodegeneration and clinical decline (Graham *et al.* 2006), possibly through prevention of nuclear entry (Warby *et al.* 2008). The autophagy pathway is capable of degrading proteins that the proteosome cannot, including mutant huntingtin aggregates, which in fact appear to activate autophagy directly (Ravikumar *et al.* 2002; Ravikumar *et al.* 2004). Further apparent effects of mutant huntingtin include impairments of endocytosis (Li *et al.* 2004), mitochondrial function (Panov *et al.* 2002) and vesicular trafficking (Zala *et al.* 2008).

Importantly, these pathogenic mechanisms have been demonstrated in neuronal cells — so-called 'cell-autonomous' effects. But as mentioned above (section I.1.4.2), far from existing in isolation, neurons are inextricably linked, both physically and functionally, with the glial cells that outnumber them by ten to one, regulate the CNS milieu, perform immune functions and modulate neurotransmission. Increasingly, far from being mere bystanders in neurodegenerative diseases, glial

cells are recognised as having critical roles in the reaction to, and progression of, neurodegeneration (Lobsiger *et al.* 2007). Support for a crucial role for microglia comes from the demonstration that the enzyme kynurenine monooxygenase (KMO), from an pathway linked to oxidative damage that is exclusive to microglia, is a therapeutic target in yeast (Giorgini *et al.* 2005).

Chapter IV of this thesis will suggest that, because of the ubiquitous expression of huntingtin across cell types, the contributions of non-neuronal cells to neuropathogenesis may be exaggerated by the effects of the mutant protein within such cells (effects that are cell-autonomous to the glial cell but non-autonomous to the neuron), amplifying their influence or even turning a protective response into a maladaptive, harmful one.

I.1.4.4 Non-CNS pathology

Conceivably, the peripheral phenotype of HD could be brought about by CNS pathology exerting indirect effects on peripheral tissues: for instance, cachexia could be brought about via endocrine effects of hypothalamic dysfunction, the autonomic nervous system or altered food intake due to behavioural change. However, though huntingtin levels are highest, and pathology most pronounced, in brain, expression of mutant huntingtin, including the formation of aggregates, has been found in every tissue where it has been sought (Strong *et al.* 1993; Sathasivam *et al.* 1999). In human patients, peripheral tissues have been less well studied but muscle displays functional, biochemical and pathological changes in HD (Arenas *et al.* 1998; Lodi *et al.* 2000) while testicular degeneration is accompanied by reduced numbers of germ cells and abnormal seminiferous tubule morphology (Van Raamsdonk *et al.* 2007), while increased density of adenosine A_{2A} receptors in brain is also seen in peripheral leukocytes (Varani *et al.* 2003; Varani *et al.* 2007).

As seen in section I.1.1.5, the peripheral cellular pathology of HD is accompanied by sometimes dramatic clinical features, and is therefore of importance in its own right. A therapy that did not enter the CNS but could halt weight loss could nonetheless bring about improvement in quality or even quantity of life even if CNS pathology remained unaltered. The peripheral pathology of HD has two other important ramifications, however. First, it is unlikely that pathology in peripheral tissue remains locally confined. Dysfunctioning and dying peripheral cells are likely to release molecules that can have distant effects on other cell types, and may directly or indirectly cross the blood-brain barrier and influence CNS pathology for better or worse. Second, even if the CNS and peripheral

processes were each confined entirely to their own compartments, the pathology in both cases begins with mutant huntingtin and is likely to share a number of fundamental features. In reality, the likely scenario is that both forms of interplay exist: that CNS and peripheral tissues share parallel pathogenic pathways, and are linked by two-way interplay across the blood-brain barrier. This model is central to the arguments presented in Chapter IV.

I.1.5 Emerging therapeutics

> *I have never known a recovery or even an amelioration of symptoms in this form of chorea; when once it begins it clings to the bitter end. No treatment seems to be of any avail, and indeed nowadays its end is so well-known to the sufferer and his friends, that medical advice is seldom sought. It seems at least to be one of the incurables.*

(Huntington 1872)

The advances in our understanding of the pathogenesis of HD since 1993 have been dramatic. Concerted and international efforts are now in place to study therapeutic targets, screen and rationally develop treatments, and test candidate disease-modifying therapies in animals and humans. There is a consensus that the coming years will see an expansion in the number of therapies shown to be effective in animal models that are put forward for clinical testing in human subjects (Handley *et al.* 2006). At the time of writing (2009), there are four registered clinical trials of putative disease-modifying therapies for HD active in the United States; a further sixteen are currently recruiting or preparing for recruitment (US National Institutes of Health 2008). This section will review the most promising approaches to disease-modification before discussing some logistical issues and introducing the role of biomarkers in HD clinical trials. While the focus of this thesis is on biomarkers and disease-modification, it should be noted that improved access to symptomatic treatments and expert multidisciplinary care are already improving the quality of life, and probably life expectancy, of HD patients and the wider availability of such interventions is a pressing imperative for healthcare provision.

I.1.5.1 Therapeutic candidates

Gene silencing refers to a number of strategies used to prevent the translation of huntingtin mRNA into the mutant protein, by means of intracellular nucleotide molecules designed to target the huntingtin message. Preventing production of the protein is clearly an appealing approach that could theoretically mitigate all the detrimental effects of the mutation. Moreover, gene silencing treatments have already shown promise in human trials in macular degeneration and respiratory syncytial virus infection and are currently in phase III trials (Arjamaa 2006; Sah 2006). Trials of RNA interference in HD animal models have shown promise, with RNA targeting mutant huntingtin delivered by viral vector injected into the striatum producing clinical and pathological improvement (Harper *et al.* 2005). Delivery and brain penetration of gene silencing therapies are challenging issues, but recent promising trials with DNA oligonucleotide therapies infused into the CSF space in other neurodegenerative diseases (Smith *et al.* 2006) suggest that they may be surmountable.

Cysteamine is an inhibitor of transglutaminases, which have been shown to be involved in the recruitment of mutant huntingtin into aggregates (Kahlem *et al.* 1998). Cystamine, a cysteamine precursor, was shown to slow progression and improve motor function in the R6/2 mouse (Dedeoglu *et al.* 2002) and is safe and well-tolerated in humans but awaits large-scale study (Dubinsky *et al.* 2006).

Histone deacetylase (HDAC) inhibitors are aimed at ameliorating transcriptional dysregulation in HD, by enhancing the availability of gene promoter sites. Suberoylanilide hydroxamic acid, an orally-administered HDAC inhibitor, produced pathological and motor improvement in the R6/2 mouse but has an unfavourable toxicity profile (Hockly *et al.* 2003). Work is underway to identify less toxic, selective HDAC inhibitors (Thomas *et al.* 2008).

Caspase inhibitors could prevent generation of toxic N-terminal fragments and nuclear entry of huntingtin. Minocycline has some caspase-inhibiting activity and has shown promise in animal studies but has yet to demonstrate efficacy in a randomised, double-blind placebo-controlled trial in human patients (Bonelli *et al.* 2004) and more potent, selective inhibitors, especially of caspase 6, are being sought.

Autophagy enhancers could improve the removal of mutant huntingtin from cells. The enzyme mTOR ('mammalian target of rapamycin') downregulates autophagy and mTOR inhibition with rapamycin reduces aggregate formation and improves motor deficits in fly and mouse models (Ravikumar *et al.* 2004). A novel mTOR-independent autophagy pathway may yield additional therapeutic targets (Williams *et al.* 2008).

Fetal stem cell transplantation has shown promise but is currently limited by technical, ethical and legislative concerns. In one study, three of five patients maintained or slightly improved motor and cognitive function after transplantation but the improvement was not sustained (Bachoud-Levi *et al.* 2006).

Microglial modulation treatments aim to mitigate the apparent harmful effects of microglial activation in HD. The KMO pathway (page 39), which was identified as a therapeutic target in yeast (Giorgini *et al.* 2005), has recently been targeted by a small-molecule inhibitor and preliminary results suggest that KMO inhibitors may be capable of dramatically improving survival in the R6/2 mouse (Muchowski 2008). Bone marrow transplantation, aimed at replacing the microglial population with wild-type cells, is also under investigation.

Mitochondrial enhancers are generally widely-available dietary supplements such as creatine, coenzyme Q and fish oil derivatives, aimed at improving the efficiency of cellular energy utilisation (Panov *et al.* 2002). Such agents have been tested in large trials and none has convincingly shown disease-modifying efficacy (e.g. Tabrizi *et al.* 2005), though enthusiasm persists for re-trialling them on a larger scale, at higher doses and in combination. Dimebon, an antihistamine with actions as an NMDA receptor antagonist and mitochondrial membrane stabiliser, has shown promise in trials of Alzheimer's disease (Doody *et al.* 2008) and clinical trials are now underway in HD.

I.2 Biomarkers for HD

I.2.1 Introduction

From the previous section, it is apparent that there is confidence that disease-modifying therapies can be developed for HD. A genetic test can reliably predict which at-risk people will develop the disease, and in such individuals there is a potentially valuable window of opportunity after genetic diagnosis during which the slowing of pathology would be expected to delay the onset of disease

symptoms. The natural history of the disease, however, raises major hurdles to the translation of potential treatments to human patients. HD progresses very slowly and causes a diverse range of signs, symptoms and rates of progression. As discussed in section I.1.1.2, the clinical rating scales used to assess progression, such as the UHDRS, are subject to intra- and inter- rater variability and lack the ability to distinguish disease modification from symptom relief. Moreover, in premanifest expansion carriers — the very group that stands to benefit most from disease-slowing treatments — there are typically for several decades no unequivocal outward signs by which to judge the success or failure of a potential therapy. Clinical trials in premanifest subjects based on current clinical outcome measures would require vast subject numbers over unfeasibly long periods (Paulsen *et al.* 2006). Reducing the sample size requirement for clinical trials is a key aim of the development of biomarkers for Huntington's disease.

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001). In many fields, such as Alzheimer's disease, biomarkers are used alongside clinical outcome measures (e.g. Fox *et al.* 2005), while in some diseases such as HIV, biomarkers are used instead of clinical measures, as surrogate endpoints (e.g. Delta Coordinating Committee 2001). Roles and characteristics of ideal biomarkers for HD are outlined in Table 2.

Roles that an ideal set of biomarkers for HD would fulfil:

- Accurate prediction of age at onset of disease manifestations;
- Prediction of rate of disease progression after onset;
- Prediction of likely clinical manifestations;
- Unequivocal diagnosis of disease onset;
- Measurement of all aspects of disease progression;
- Determination that a putative treatment has reached and activated its target (pharmacodynamic biomarkers);
- Assessment of efficacy of putative disease-modifying interventions.

Characteristics of an ideal biomarker (after Henley *et al.* 2005):

- Quantifiable reliably, reproducibly and minimally invasively;
- Subject to minimal variation in the general population;
- Unaffected by comorbidity or symptomatic medication;
- Linearly altered by disease progression;
- Subject to predictable alteration when the disease is modified by an intervention

Table 2 Potential roles and ideal characteristics of biomarkers for HD

The more closely a biomarker is mechanistically linked to disease pathogenesis — and the better understood the links are — the more likely it is to fulfil the final two criteria.

A conceptual outline for the evaluation of the potential utility of candidate biomarkers is shown in Figure 10.

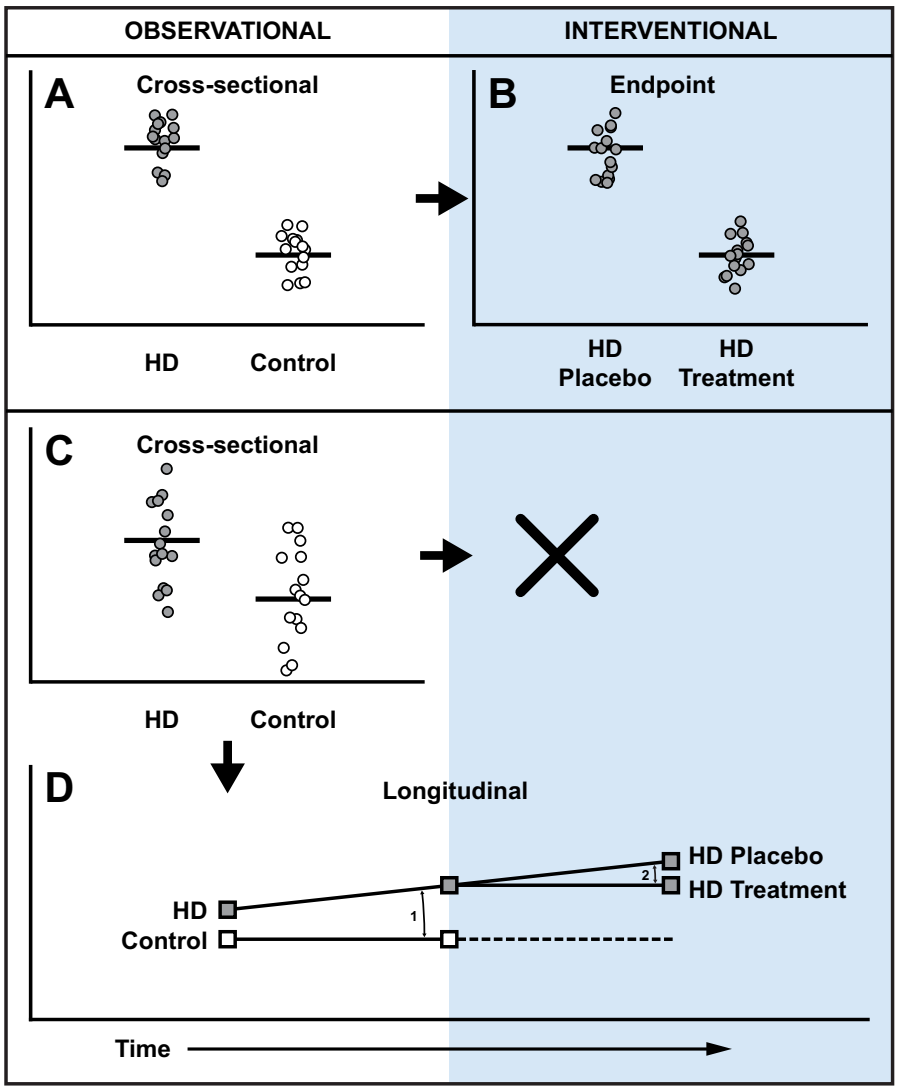

Figure 10 Outline of the evaluation of the utility of potential biomarkers

*Ideal biomarker candidates may achieve complete separation cross-sectionally between HD patients and controls (**A**). If a successful treatment could be expected to return the biomarker to control levels, such a marker may be considered for use directly as a surrogate endpoint in an interventional trial (**B**). Some biofluid markers may fall into this category. If there is incomplete separation cross-sectionally (**C**), or if a successful treatment would not be expected to return the biomarker to control levels, it is necessary to study the biomarker in patients and controls longitudinally (**D**), to establish that the rate of change in patients is significantly different from that in controls. If it is (1), the marker may then be used longitudinally in an interventional trial, the surrogate endpoint being the alteration of the rate of change in treated patients towards the rate previously observed in controls (2). Most imaging and quantitative motor markers will likely fall into this category.*

To date, disease-modification trials in HD have generally been of low-potency agents such as dietary supplements, tested in cohorts of manifest HD subjects, monitored using clinical endpoints, and have had negative outcomes (e.g. Tabrizi *et al.* 2003; Tabrizi *et al.* 2005; Hersch *et al.* 2006; e.g. Biglan *et al.* 2007; Amarin Neuroscience Ltd *et al.* 2008). Where biomarkers have been employed in such trials it has been as pharmacodynamic markers of drug entry to the CNS or target occupancy (Bender *et al.* 2005; Hersch *et al.* 2006).

Until markers of progression can be found for premanifest individuals, treatment trials of agents expected to influence disease progression are likely to continue to take place in manifest HD, because of the established role of clinical endpoints and need for clear links between biomarkers and 'hard' clinical endpoints (Figure 11). The demonstration that the clinical and pathological phenotype can be reversed in adulthood in HD mice if the expression of mutant huntingtin is stopped (Yamamoto *et al.* 2000) reinforces the validity of this approach, since a putative treatment that did not at least slow clinical progression in a suitably powered clinical trial could reasonably be deemed unlikely to be disease-modifying. Clinical measures may then be used as primary endpoints with candidate biomarkers employed in parallel as secondary endpoints, following the model of other diseases such as HIV (Delta Coordinating Committee and Virology Group 1999; Delta Coordinating Committee 2001) and Alzheimer's disease (Fox *et al.* 2005), enabling the parallel evaluation of biomarker candidates, the assessment of therapies and the investigation of the relationships between disease, biomarker and therapy.

The ultimate aim of HD therapeutics development is to identify treatments that can be shown to ameliorate the very earliest pathological changes in premanifest disease, delaying clinical onset and securing additional years of impairment-free living, in addition to increased life expectancy (Figure 11). Once this has been achieved, it becomes merely a qualitative matter to defer clinical onset to a time beyond the individual's natural life expectancy, amounting to a *de facto* cure. Such treatment of premanifest individuals has two prerequisites: first, a panel of biomarkers capable of predicting onset and measuring the efficacy of treatments in a manner that predicts clinical outcomes; and second, a treatment that interacts as expected with both pathobiology and biomarkers, with a favourable profile of benefit to adverse effects so as to ensure long-term compliance.

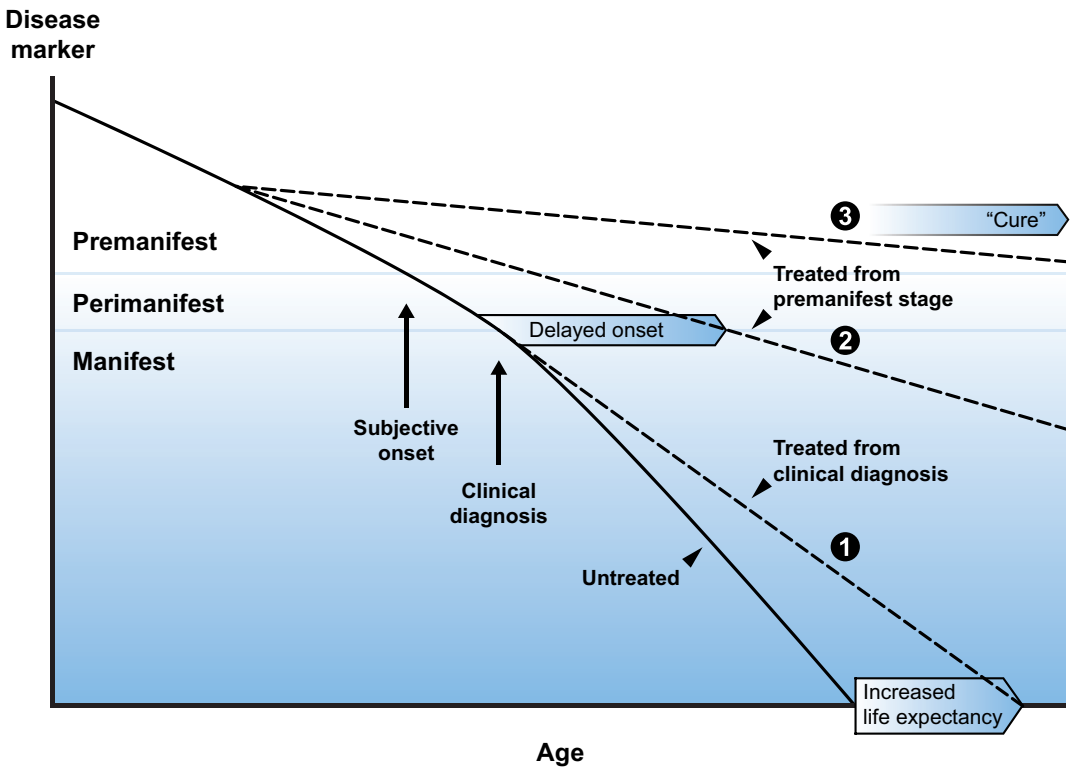

Figure 11 Schematic showing the likely evolution of disease-modifying therapies for HD

The disease trajectory is shown in terms of a putative disease marker (vertical axis) capable of tracking progression from premanifest disease to death. The earliest treatments (❶) are likely to be licensed for initiation in manifest disease. Such an approach would increase life expectancy and is likely to improve quality of life overall, with the caveat that patients will inevitably be sustained in a state where they have a degree of impairment. If treatments can be demonstrated to be effective in slowing pathology, and sufficiently safe for administration to premanifest gene carriers, much more favourable outcomes will likely result, from deferring clinical onset and hence securing extra impairment-free years, in addition to increased life expectancy (❷) to, ultimately, a deferral of disease onset to beyond the individual's natural life expectancy — a de facto *cure (❸).*

This section will review the current state of biomarker research in HD, focusing on the four areas most likely to produce useful biomarkers for HD: neuroimaging, biofluids, quantitative motor assessment and cognitive measures. It concludes with a discussion of the substantial remaining challenges to the conduct of biomarker-powered treatment trials in HD.

I.2.2 Imaging

I.2.2.1 Introduction

Modern neuroimaging techniques such as magnetic resonance imaging (MRI) enable high quality images of brain structure and function to be obtained, while advanced image analysis techniques allow robust measurement of differences between subjects and change within individuals. As well as being relatively non-invasive, its clear relevance to neuropathology (Fox *et al.* 1996) makes neuroimaging an appealing source of biomarkers.

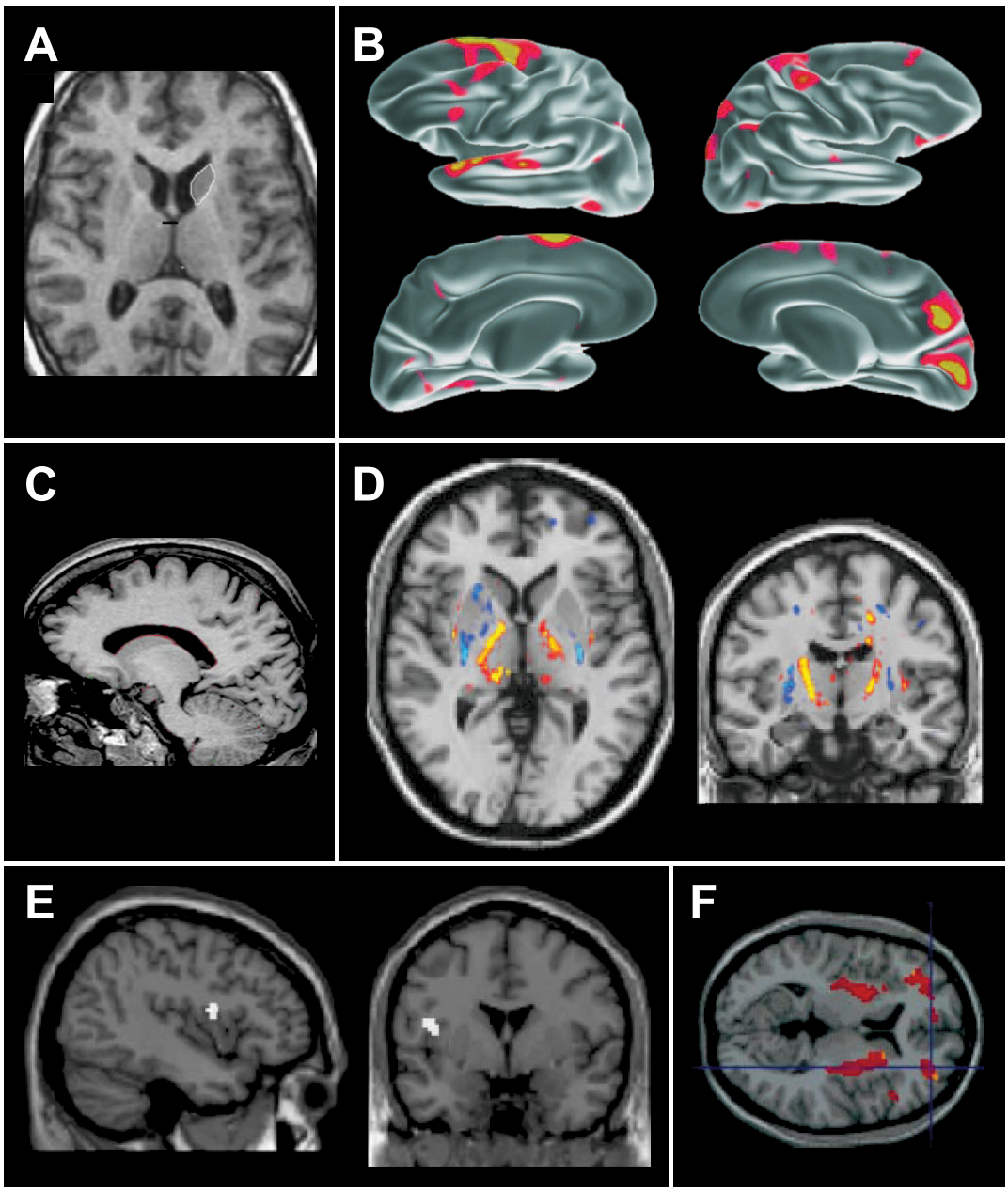

Figure 12 Potential imaging biomarkers for HD

A. *Volumetric assessment of basal ganglia structures (Aylward* et al. *2003).* ***B***. *Cortical thickness measurement showing regions of cortical thinning in premanifest HD (Rosas* et al. *2005).* ***C***. *Measurement of whole brain atrophy using the boundary shift integral. Boundary regions undergoing atrophy in early HD are highlighted in red (Henley* et al. *2006).* ***D***. *Diffusion tensor imaging fractional anisotropy map showing areas of increased (red-yellow) and decreased (blue) white matter organisation in premanifest HD (Rosas* et al. *2006).* ***E***. *Functional MRI map showing regionally*

decreased activation in premanifest HD in response to disgusted facial expressions (Hennenlotter et al. *2004).* ***F****. Positron-emission tomography (PET) map showing regions where D2 dopamine receptor binding decreased significantly over 29 months (Pavese* et al. *2003).*

I.2.2.2 Serial volumetric MRI

Volumetric imaging enables the volumes of brain regions to be estimated. T1 volumetric MRI is the most established technique in HD.

I.2.2.2.1 Striatal morphometry

The GABAergic medium spiny neurons of the striatum have long been known to bear the burden of cell death in early Huntington's disease (Bates *et al.* 2002). The basal ganglia have extensive cortical connections that could account for the motor, cognitive and behavioural dysfunction of the disease (Alexander *et al.* 1986).

Basal ganglia structures have been shown, by manual outlining, to have reduced volume in early HD (Harris *et al.* 1992) and premanifest disease (Aylward *et al.* 1994) (Figure 12A). Cross-sectional caudate volume has been shown to correlate with estimated disease onset probability based on CAG repeat length (Aylward *et al.* 1996) and performance on some cognitive tests (Brandt *et al.* 1995).

Because of overlap between HD patients and controls, and the fact that even a successful intervention may not restore lost brain volume (see Figure 10), cross-sectional volumetric imaging may be less likely to provide useful biomarkers than longitudinal measurement of brain volume loss. Studies using volume subtraction of manually segmented caudates have demonstrated significantly faster atrophy in early disease (Aylward *et al.* 1997) and in premanifest gene carriers as far as 11 years from predicted onset, over a mean inter-scan interval of 4 years (Aylward *et al.* 2000; Aylward *et al.* 2004). More recent exploratory studies have proposed automated techniques for improving the reliability of regional atrophy measures (Rosas *et al.* 2001; Hobbs *et al.* 2007).

I.2.2.2.2 Cortical and whole-brain morphometry

Although basal ganglia structures are affected by atrophy relatively more than other brain regions, measurement of a small change in a large volume, rather than a large fractional change in a small region, may, if the measurements are more precise, generate data less susceptible to segmentation

inaccuracy. The majority of atrophy in HD is extrastriatal (see section I.1.4.1) and the relative contributions to disease manifestations from striatum and cortex are not known, so treatments aimed at preserving striatal function may fail to ameliorate the disease (Rosas *et al.* 2004); conversely, disease modification brought about by a treatment aimed at cortical function may be missed by measures of striatal atrophy.

In cross-sectional studies, Rosas and colleagues have confirmed by a semi-automated technique that numerous extrastriatal brain areas are atrophied in HD (Rosas *et al.* 2003). They have used an automated technique to demonstrate cortical thinning cross-sectionally in early HD (Rosas *et al.* 2002) and premanifest gene carriers (Rosas *et al.* 2005) that correlates with changes in cognition (Figure 12B). Longitudinal studies of cortical thinning in HD are underway.

The boundary shift integral (BSI) is a semi-automated method by which changes in brain volume can be calculated from registered scan pairs. Semi-automated segmentation of the baseline region is followed by automated calculation of the volume change based on the movement of its boundary. It has been shown to be more reliable than simple volume subtraction (Freeborough *et al.* 1997) and is widely used in observational and interventional trials of Alzheimer's disease (Fox *et al.* 2005). Using the BSI, Henley and colleagues demonstrated that whole-brain atrophy was significantly faster in early HD than controls, over a short 6-month period (Henley *et al.* 2006) (Figure 10C).

Voxel-based morphometry (VBM) and tensor-based morphometry (TBM) are automated techniques for analysing regional morphological differences between groups of MRI scans. Cross-sectionally, VBM has identified brain regions differentially affected between different disease groups and controls (Thieben *et al.* 2002; Kassubek *et al.* 2004; Kassubek *et al.* 2005) but it has not yet been applied longitudinally in HD. TBM has demonstrated increased atrophy over 2 years in premanifest HD (Kipps *et al.* 2005). Though the insights from these techniques are useful in enhancing our understanding of HD and directing subsequent regional atrophy studies, some loss of statistical power is inevitable when from deriving regionalising information from whole-brain comparisons without pre-defined regions of interest. As such, the main value of VBM and similar techniques may be in identifying regions of interest for directed study as potential biomarkers.

I.2.2.3 Diffusion tensor imaging

Diffusion tensor imaging (DTI) uses the ability of water to diffuse along axons to produce maps of white matter fibre tracts and quantitative measures of damage to axonal integrity. It can detect abnormalities in disorders such as multiple sclerosis where conventional MRI scans appear normal (Werring *et al.* 1999). Consequently DTI is appealing as a potential means of measuring neuropathology earlier in HD than other techniques.

Reading and colleagues identified several regions of decreased fractional isotropy (FA, a measure of axonal organisation) compared with controls in premanifest HD (Reading *et al.* 2005). Rosas and colleagues demonstrated several regions where FA correlated with cognitive performance in premanifest HD and found more widespread abnormalities in manifest HD (Rosas *et al.* 2006) (Figure 10D). Klöppel and colleagues used DTI tractography to measure connections between the basal ganglia and frontal cortex, enabling the correct classification of 82% of scans as coming from premanifest HD subjects or controls. They further demonstrated that the loss of such fibres correlates with impairment of saccadic eye movements in HD (see also section I.2.4.1) (Klöppel *et al.* 2008).

DTI shows promise as a potential biomarker capable of detecting change in HD earlier than other imaging measures. As a relatively new technique, however, its robustness remains to be tested across populations and longitudinally in HD.

I.2.2.4 Functional MRI

Functional MRI (fMRI) uses changes in regional blood flow during neuronal activity to identify brain regions active during performance of experimental tasks. It has the potential to reveal early abnormalities in HD due to neuronal dysfunction. Because neuronal dysfunction is more likely to be reversible than cell death, fMRI measures may not require longitudinal measurement to be useful as surrogate endpoints (see Figure 10).

Several fMRI studies have demonstrated regional functional abnormalities in early HD (Clark *et al.* 2002; Aron *et al.* 2003; Thiruvady *et al.* 2007). In premanifest subjects, region-specific alterations in functional activity have been shown to correlate with estimated time to onset (Hennenlotter *et al.* 2004; Paulsen *et al.* 2004). One study comparing volumetric and functional MRI data found that

regions with altered activity were not those experiencing the most atrophy, suggesting that fMRI abnormalities may reflect dysfunction in basal ganglia output pathways or recruitment of secondary populations of neurons (Gavazzi *et al.* 2007).

While fMRI is appealing as a marker of neuronal dysfunction in HD, there are more technical hurdles to its adoption as a biomarker for clinical trials. The equipment and expertise to perform fMRI studies are less widespread than conventional MRI techniques and scanning times are typically longer. fMRI is highly susceptible to apparently trivial differences in conditions (such as ambient light or sound levels) and therefore difficult to standardise across sites as required for large clinical trials. It is unknown to what extent observed fMRI changes reflect of neuropathology *per se*, rather than psychological factors in patients (e.g. performance anxiety in subjects who know they are destined to develop HD).

I.2.2.5 MR Spectroscopy

MR spectroscopy (MRS) can non-invasively quantify the biochemical composition of tissues including brain. Lactate levels have been shown to be elevated in the striatum in premanifest disease (Jenkins *et al.* 1998; Reynolds *et al.* 2005) and early HD (Koroshetz *et al.* 1997; Jenkins *et al.* 1998; Reynolds *et al.* 2005) and appear to respond to treatment with coenzyme Q10, a treatment aimed at brain energy metabolism (Koroshetz *et al.* 1997). However, the finding of increased lactate has not been reproduced across all studies (Hoang *et al.* 1998). MRS has identified cross-sectional changes in N-acetylaspartate and creatine (Sanchez-Pernaute *et al.* 1999; Reynolds *et al.* 2005) and in one study, these changes were shown to correlate with motor dysfunction (Sanchez-Pernaute *et al.* 1999). A sequence of metabolite changes may be detectable with disease progression, with N-acetylaspartate reductions the earliest feature, giving way to reduced creatine followed by elevation of glutamate and lactate after motor onset (Reynolds *et al.* 2005). More recently, MRS has been used in a trial of creatine therapy to demonstrate that the compound entered the brain — that is, as a pharmacodynamic biomarker (Hersch *et al.* 2006). The utility of MRS is limited by long scan times, the small number of molecules it can accurately detect, its relative insensitivity and the use of metabolite ratios which may obscure individual metabolite changes. Nonetheless, its ability to glimpse the biochemical milieu of the central nervous system gives it promise as a source of pharmacodynamic biomarkers in HD.

I.2.2.6 Positron-emission Tomography

Positron-emission tomography (PET) uses positron emission by radioisotopes to identify regional functional variations *in vivo*. While fMRI has largely supplanted PET imaging in the visualisation of regional brain activity, the ability of PET to highlight receptor density selectively by the use of radiolabelled ligands is of value.

Post-mortem studies have shown 50% loss of dopamine receptors in the striatum by end-stage HD (Cross *et al.* 1983). Dopamine receptors have been widely studied using the PET ligands ^{11}C-raclopride (which binds D_2 receptors) and ^{11}C-SCH23390 (which binds D_1 receptors). Dramatically reduced D_1 and D_2 binding is seed in manifest HD (Turjanski *et al.* 1995) and correlates with the duration of motor manifestations (Ginovart *et al.* 1997). In premanifest HD, reductions in dopamine receptor binding are more variable but significant (Weeks *et al.* 1996) and correlate with CAG repeat length (Antonini *et al.* 1998), estimated time to motor onset (van Oostrom *et al.* 2005) and subtle cognitive changes (Lawrence *et al.* 1998). Longitudinal studies have shown consistent decreases in D_2 binding of around 5-6% annually in manifest disease (Pavese *et al.* 2003) and around 4% in premanifest disease, with faster rates of loss in those approaching motor onset (Andrews *et al.* 1999), suggesting that ^{11}C-raclopride binding may detect both progression and incipient motor onset. Recently ^{11}C-raclopride binding has been used to monitor fetal striatal grafts (Furtado *et al.* 2005). One potential weakness of dopamine receptor binding studies using PET is that they are more sensitive to striatal than extrastriatal pathology. However, statistical parametric mapping has recently shown that there is also loss of D_2 receptor binding in the other brain regions (Pavese *et al.* 2003).

Microglial activation is seen post-mortem in HD brain (Messmer *et al.* 1998; Sapp *et al.* 2001) and microglia have been implicated as a potential source of tissue damage in HD (Giorgini *et al.* 2005). The PET ligand ^{11}C-R-PK11195 binds benzodiazepine receptors expressed selectively by activated microglia. It has demonstrated increased microglial activation in the striatum in manifest HD that correlates with reduced ^{11}C-raclopride binding, UHDRS motor scores and CAG repeat length (Pavese *et al.* 2006). Microglial activation is also seen in premanifest disease where it correlates with onset probability (Tai *et al.* 2007). These findings suggest that microglial activation is an early and consistent marker of HD pathogenesis.

PET scanning offers early visualisation and quantification of pathological abnormalities in HD, and thus shows promise as a source of biomarkers in HD. PET imaging is already used as a biomarker clinically and in disease-modifying trials in Alzheimer's disease (Nordberg 2007). However, a number of drawbacks limit its utility in HD: the cost of PET scanning is very high and the availability of PET scanning equipment and expertise is limited; the radioactive ligands are potentially hazardous and difficult to prepare and manipulate; scans are time-consuming; and, being ligand-based, PET scanning is susceptible to influence by medications commonly used in HD (such as neuroleptics, which bind D_2 receptors).

I.2.3 Biofluid biomarkers

I.2.3.1 Introduction

Molecular biomarkers that can be quantified in biofluids such as blood or urine are appealing because of the minimal requirement for patient involvement, opportunity for rapid bulk processing of specimens, availability of reliable assays and because several analyses can be carried out on a single sample. Approaches to the discovery of potential markers have ranged from hypothesis-driven searches investigating pathways known to be deranged in HD (e.g. Hersch *et al.* 2006; e.g. Ciammola *et al.* 2007), to the application of 'omics' technologies to find differences between HD patients and controls (e.g. Borovecki *et al.* 2005; Dalrymple *et al.* 2007; Runne *et al.* 2007).

The ideal peripheral blood biomarker of HD would be a direct product of neuronal dysfunction, normally absent from blood, that leaked across the blood-brain barrier and became detectable in blood. However, attempts to find such markers in blood have hitherto been unsuccessful. Most of the potential biomarkers studied to date are likely wholly or largely produced by peripheral tissues as a result of effects of the ubiquitously-expressed mutant huntingtin. As discussed in section I.1.4.4, some such changes may represent peripheral processes that recapitulate CNS pathogenic pathways. In other cases, signalling molecules may cross the blood-brain barrier from the CNS and produce peripheral changes. These mechanistic differences will inevitably influence the utility of each potential marker: for instance, a peripherally produced marker that reflects CNS pathogenesis, but does not measure it directly, would not be useful as a pharmacodynamic marker of CNS target occupancy but may be useful for prediction of disease onset or response to treatment. This

underscores the importance of understanding the place of each potential marker with respect to the pathogenic pathways of HD.

Another problem universal to biomarker identification that particularly affects biofluid studies is the possible effect of medications. Ideally, biomarker discovery work would be performed using only unmedicated subjects. However, such patients are likely to be unrepresentative of the HD population and the results of such studies may not be applicable to the many subjects taking medications for symptomatic relief or concomitant illnesses, who would need to be included in therapeutic clinical trials. It may therefore be more practicable to ensure that medications are recorded and attempts made to identify and account for possible medication artefacts on biomarker discovery.

The potential biofluid biomarkers reported to date are summarised in Table 3.

Marker	Alteration	Tissue	Key reference
Oxidative stress			
8OH2′dG	Increased	Serum	(Hersch *et al.* 2006)
Malondialdehyde	Increased	Plasma	(Chen *et al.* 2007)
GPx and Cu/Zn-SOD	Reduced	Erythrocytes	(Chen *et al.* 2007)
Metabolic markers			
Panel of 17 metabolites	Mixed	Serum	(Underwood *et al.* 2006)
Valine, leucine and isoleucine	Reduced	Plasma	(Mochel 2007)
Cholesterol biosynthetic pathway	Reduced	Cultured cells	(Valenza *et al.* 2005)
Transcriptomic markers			
Panel of 12 transcripts	Increased	Blood	(Borovecki *et al.* 2005)
IER3	Increased	Blood	(Runne *et al.* 2007)
Signalling pathways			
BDNF	Reduced	Serum	(Ciammola *et al.* 2007)
FAAH	Reduced	Lymphocytes	(Battista *et al.* 2007)
Anandamide	Increased	Lymphocytes	(Battista *et al.* 2007)
A2A receptors	Increased	Lymphocytes	(Varani *et al.* 2007)
Endocrine markers			
Cortisol	Increased	Urine	(Björkqvist *et al.* 2006)
Ghrelin	Increased	Plasma	(Popovic *et al.* 2004)
Leptin	Decreased	Plasma	(Runne *et al.* 2007)
Testosterone (males only)	Decreased	Plasma	(Markianos *et al.* 2005)
CART	Increased	CSF	(Björkqvist *et al.* 2007)

Table 3 Summary of published biofluid biomarker candidates identified to date in HD

8OH2′dG, 8-hydroxy-2-deoxy-guanosine; GPx, glutathione peroxidase; Cu/Zn-SOD, copper-zinc superoxide dismutase; IER3, immediate early response 3; BDNF, brain-derived neurotrophic factor; FAAH, fatty acid amide hydrolase; IL6, interleukin 6; CART, cocaine- and amphetamine-regulated transcript.

I.2.3.2 Markers of oxidative stress

8-hydroxy-2-deoxyguanosine (8OH2'dG) is formed when DNA is subjected to oxidative damage. It has been shown to be increased in brain, urine and plasma of amyotrophic lateral sclerosis patients and HD mice (Bogdanov *et al.* 2001). Serum levels in HD were found to be dramatically elevated compared with controls and fell significantly in a group of patients given the antioxidant treatment creatine rather than a placebo (Hersch *et al.* 2006). Since 8OH2'dG levels could be reduced directly by the antioxidant effect of creatine, rather than by any effect on huntingtin-mediated toxicity, in this setting 8OH2'dG is a pharmacodynamic marker. This clear separation of patients from control subjects (as in Figure 10A) is promising, but a recent attempt to reproduce it in another population was unsuccessful, possibly because of baseline differences in consumption of antioxidant supplements (Biglan *et al.* 2007).

Another group examining oxidative damage found increased 8OH2'dG levels in HD leukocytes but did not measure serum levels. They also found increases in plasma malondialdehyde, a marker of lipid peroxidation, reduced activity of two erythrocyte enzymes (GPx and Cu/Zn-SOD) and abnormalities of mitochondrial DNA (mtDNA) (Chen *et al.* 2007). Oxidative damage to mtDNA in HD has recently been shown by another group (Liu *et al.* 2007).

I.2.3.3 Metabolic markers

Huntingtin is ubiquitously expressed (Sathasivam *et al.* 1999) and, in addition to neurological features, the peripheral phenotype of HD includes weight loss and there may be an energy utilisation deficit (Farrer *et al.* 1985; Mochel 2007). Two groups have used metabolic profiling to examine HD-associated differences in metabolite levels in peripheral blood.

Underwood and colleagues used gas chromatography / time-of-flight / mass spectrometry (GC-TOF-MS) to quantify the levels of 1275 metabolites in serum from humans and R6/2 mice, followed by multivariate statistical modelling to identify the most consistently altered metabolites. They identified a pro-catabolic pattern of changes (see Table 4) which was similar in both mice and humans, and present even in premanifest subjects (Underwood *et al.* 2006).

Metabolite	Function
Glycerol	Product of triglyceride breakdown
Monosaccharides	Carbohydrate metabolism
Lactate	Anaerobic metabolism
Alanine	Amino acid
Leucine	Branched chain amino acid
2-amino-n-butyrate	Intermediate in pyrimidine metabolism
Ethylene glycol	Glycerol metabolite
Alpha-hydroxybutyric acid	Unknown
Valine	Branched chain amino acid
Monosaccharide	Carbohydrate metabolism
Malonate	Unknown
Urea	Nitrogen excretion

Table 4 Metabolites identified by GC-TOF-MS as contributing, with 5 unidentified others, to the signal discriminating HD patients from controls

(Underwood et al. *2006.) The branched chain amino acids leucine and valine were found by ^{1}H-NMR to be downregulated in a separate population (Mochel 2007).*

Using proton nuclear magnetic resonance (NMR) spectroscopy to quantify a different subset of metabolites, Mochel and colleagues found that controls, premanifest gene carriers, and patients at different disease stages were distinguishable by decreasing levels of the branched chain amino acids (BCAA) valine, leucine and isoleucine (Mochel 2007). These findings echoed the results of much earlier metabolic studies (e.g. Phillipson *et al.* 1977) and more recent work by others (Underwood *et al.* 2006), but in the Mochel study, BCAA levels were found to correlate with UHDRS motor scores, CAG repeat length and retrospectively determined weight loss, suggesting a hypermetabolic state early in HD. Possible effects of medications were not accounted for in this study and its findings have yet to be validated in a second population (Mochel 2007).

Another group investigating the cholesterol biosynthetic pathway in human HD cells derived from peripheral blood identified reductions in sterol regulatory element-binding proteins, which regulate

the pathway (Valenza *et al.* 2005). The techniques used to isolate and study the cells in this study would be difficult to apply to large cohort studies but the pathway is of interest in future biomarker work.

I.2.3.4 Transcriptomic markers

Transcriptional dysregulation is a feature of HD (Cha 2000) and thus it is reasonable to expect altered gene transcription that is detectable outside the central nervous system. One group identified transcriptional changes in muscle in HD patients consistent with a change in fibre type (Strand *et al.* 2005). However, muscle is a relatively inaccessible tissue and serial biopsies for biomarker analysis in clinical trials are unlikely to be feasible.

Borovecki and colleagues used microarray profiling in whole blood to identify changes in gene expression. They identified a panel of 12 genes whose expression was significantly altered from controls and across disease stages, including early and late premanifest patients based on predicted onset. These changes were confirmed in a test set of samples and shown to respond to treatment with phenylbutyrate, a histone deacetylase inhibitor affecting transcriptional regulation that is neuroprotective in HD mice (Borovecki *et al.* 2005). However, when Runne and colleagues subjected blood samples from another population of HD patients to similar analysis, they found no significant changes in the 12 genes identified by Borovecki *et al.* This finding may be explained by genetic, environmental, medication or dietary differences between populations or the inherent statistical and technical challenges of transcriptomics. Indeed, when Runne and colleagues performed further discovery and evaluation experiments using both transcriptomic and pathway-based approaches, they only identified one RNA (IER3) that was significantly upregulated in HD, and the difference in expression was modest (Runne *et al.* 2007). The apparent failure of gene expression profiling in blood to produce readily measured biomarkers is disappointing, but as the maturing technology is applied to different patient populations and in longitudinal studies, useful transcriptomic biomarkers may yet emerge.

I.2.3.5 Signalling pathways

I.2.3.5.1 BDNF

Brain-derived neurotrophic factor (BDNF) is a growth factor that promotes survival of nerve cells, including striatal neurons, during development and after injury. BDNF levels are reduced in HD

brain, particularly in the striatum (Ferrer *et al.* 2000). BDNF concentrations are lower in serum in HD animal models, and increased by treatment with the neuroprotective agents cystamine and cysteamine (Borrell-Pages *et al.* 2006). Significantly decreased BDNF levels were found in the serum of symptomatic HD patients (Ciammola *et al.* 2007) and serum BDNF is certainly interesting as a potential marker of progression. However, though BDNF does cross the blood-brain barrier, serum BDNF is largely derived from platelets, which are thought to acquire it from a number of sources including brain, peripheral nerve and vascular endothelium (Lommatzsch *et al.* 2005). The balance of central and peripheral contributions to altered serum BDNF levels in HD requires further study.

I.2.3.5.2 Endocannabinoid system

In a study examining the endocannabinoid system, which has been shown to be downregulated in the brain of R6/2 mice (Lastres-Becker *et al.* 2003), the activity of the enzyme fatty acid amide hydrolase (FAAH) in HD lymphocytes was decreased to 10% of control levels. Levels of its substrate, endocannabinoid anandamide, were correspondingly higher. These abnormalities were seen in both premanifest and manifest HD (Battista *et al.* 2007).

I.2.3.5.3 Adenosine receptors

Adenosine A_{2A} receptors are expressed on striatal medium spiny neurons and peripheral blood cells, and are dysfunctional in HD cell models (Varani *et al.* 2001). In HD leukocytes, A_{2A} receptors were shown to have increased density and affinity in manifest disease (Varani *et al.* 2003) and premanifest carriers (Varani *et al.* 2007) but no correlation with CAG repeat length or advancing disease stage has been shown.

I.2.3.6 Endocrine markers

Several features of HD, including depression, sleep disturbance and weight loss are also seen in hypothalamic dysfunction, and cell loss occurs in the hypothalamus in HD (Petersen *et al.* 2006). Several groups have identified alterations of endocrine function that may track with disease progression.

The R6/2 mouse demonstrates several features of an overactive hypothalamic-pituitary-adrenal axis and urinary cortisol levels increase progressively with advancing HD in humans (Björkqvist *et al.* 2006). Plasma (but not CSF) levels of the appetite-regulating hormones ghrelin and leptin were

respectively increased and decreased in HD, a pattern suggesting endocrine compensation for negative energy balance (Popovic *et al.* 2004). Lower plasma testosterone levels are seen in male, but not female, HD patients, and levels correlate with several cognitive scores (Markianos *et al.* 2005; Markianos *et al.* 2007). Cocaine- and amphetamine-regulated transcript, a neuropeptide with that modulates appetite and mood, was shown to be increased in CSF in HD (Björkqvist *et al.* 2007).

These endocrine changes are of interest neuropathologically and clinically. They may also yield useful biomarkers. Some endocrine abnormalities such as hypotestosteronism may be amenable to treatment using existing therapies, which may have both central and peripheral benefits. If treatable, measures of endocrine dysfunction will become biomarkers of pharmacodynamics rather than of progression. Endocrine features are highly susceptible to influences other than pathology, such as drugs and depression; this must be borne in mind when assessing their utility as potential biomarkers.

I.2.4 Quantitative clinical measures

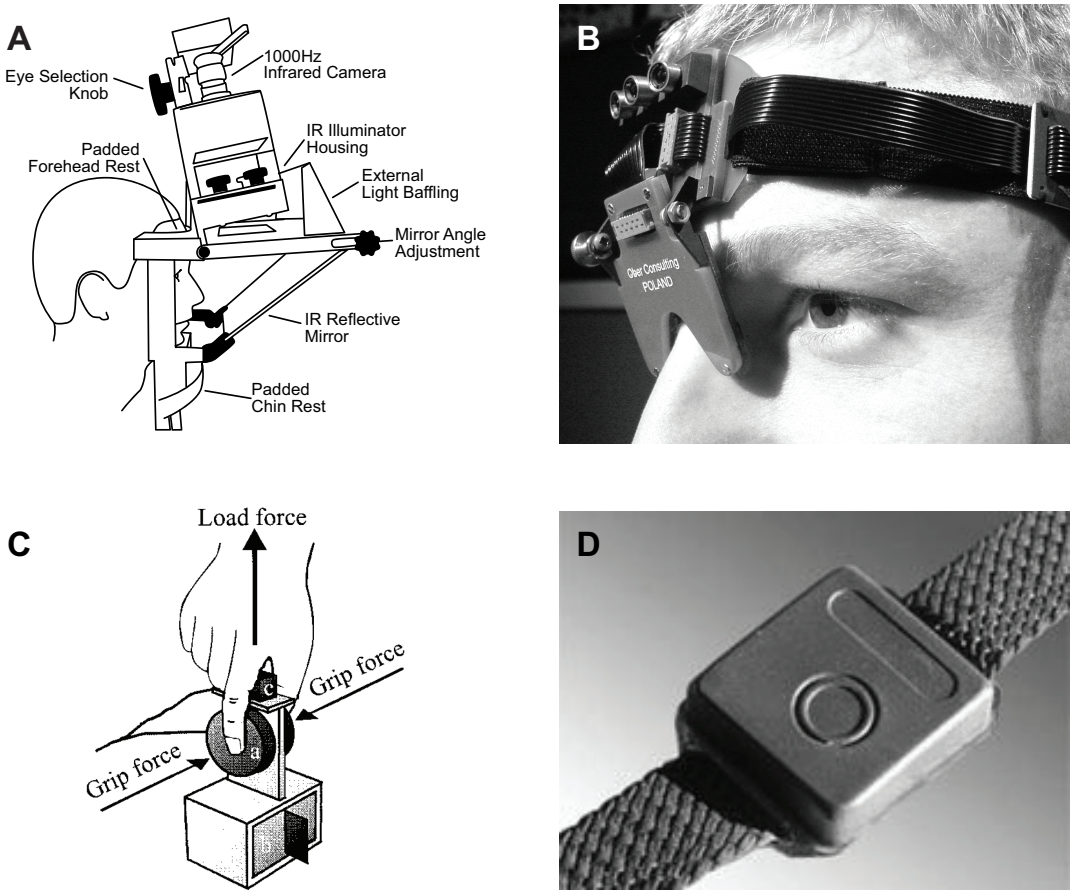

Figure 13 Some quantitative motor assessment tools proposed as biomarkers for Huntington's disease

A. *Desk-mounted equipment for oculomotor assessment (e.g. Golding* et al. *2006).* ***B***. *Portable, forehead-mounted saccadometer (Ali* et al. *2006).* ***C***. *Apparatus for measurement of grip force (Gordon* et al. *2000).* ***D***. *Wrist-mounted actigraphy device (Hurelbrink* et al. *2005).*

Clinical rating scales such as the UHDRS (The Huntington Study Group 1996) are the current standard for the assessment of progression in Huntington's disease. However, even in experienced hands, all clinical measures are subject to inter-rater variability which contributes, along with the imperfect ability of motor measures to capture disease progression, to the large sample size requirements for clinical trials based on such measures (Paulsen *et al.* 2006). Attention has therefore focused on automated techniques aimed at detecting subtle motor abnormalities earlier in premanifest HD and increasing the reliability of motor measures in manifest disease.

I.2.4.1 Oculomotor assessment

Disorders of eye movements are an established and consistent feature of HD (Bates *et al.* 2002). Oculomotor abnormalities are subtle, and therefore difficult to assess and quantify accurately. Nonetheless, because of the involvement of frontal-striatal circuits in oculomotor control, eye movement abnormalities are an appealing source of insight into pathways implicated in premanifest and early HD.

Computerised quantitative eye movement measurement has demonstrated a number of reproducible oculomotor abnormalities in HD that may function as biomarkers. In manifest HD, saccade initiation is delayed and the ability to repress reflexive saccades is impaired; in premanifest HD, subtle abnormalities were identified in saccade initiation and accuracy of memory-guided saccades (Blekher *et al.* 2004). Golding and colleagues demonstrated that voluntary saccade initiation delay correlated with disease stage in manifest HD, and estimated pathological disease burden in premanifest subjects (Golding *et al.* 2006). These findings were demonstrated using expensive specialist eye-tracking equipment (see Figure 13A). Recently developed, inexpensive, portable 'saccadometer' devices (see Figure 10B) may greatly facilitate the assessment of oculomotor dysfunction as a biomarker and its integration into multi-centre clinical trials. Early results from these devices in HD have been promising (Ali *et al.* 2006).

As mentioned in section I.2.4.1, a recent multimodal approach by Klöppel and colleagues combined automated oculomotor examination and DTI fibre tracking, affirming the neuroanatomical basis for both oculomotor assessment and DTI in tracking HD progression (Klöppel *et al.* 2008).

I.2.4.2 Tapping tests

Semi-quantitative finger-tapping assessment forms part of the UHDRS motor examination (The Huntington Study Group 1996), but tapping tasks are amenable to automated measurement and analysis.

In one paradigm, subjects were instructed to tap as quickly as possible on a sensor. Tapping rates were significantly lower than controls in manifest HD patients but not premanifest gene carriers. Rates correlated with UHDRS motor scores and HD duration, but not with CAG repeat length (Saft *et al.* 2006). Longitudinally tapping rates declined significantly over 3 years in manifest HD, but this

interval was longer than required for the UHDRS motor score to increase significantly, indicating that tapping was less sensitive at detecting progression than the UHDRS motor score (Andrich *et al.* 2007).

Tasks of rhythmic tapping, where subjects tap at regular intervals with or without an auditory pacing signal, have shown more promise as a potential marker for motor onset emerging among the most promising psychomotor tasks from the baseline analysis of the Predict-HD study, where accuracy of unpaced tapping and variability of paced tapping correlated with the 'soft' motor signs that herald onset (Paulsen *et al.* 2006). Variability of paced tapping also correlated with probability of motor onset estimated from age and CAG length (Hinton *et al.* 2007). Longitudinal data from Predict-HD on tapping measures are awaited.

I.2.4.3 Grip force

Assessment of grip force is a test of non-repetitive voluntary movement encompassing sensorimotor integration and motor persistence. In a cross-sectional study, automated measurement (Figure 13C) revealed greater variability in force during static gripping in HD patients than controls that correlated with total functional capacity and cognitive scores (Gordon *et al.* 2000). Longitudinally, grip force variability increased in 100% of subjects over 3 years (Reilmann *et al.* 2001). Preliminary data suggest increased grip force variability in premanifest HD (Reilmann *et al.* 2007).

I.2.4.4 Actigraphy

Several wrist-mounted devices (see Figure 13D) can produce long-term recordings of subjects' overall activity (actigraphy) and have been studied in HD. In one study, 5-day recordings demonstrated reduced activity cross-sectionally in HD patients compared with controls, and no significant change in activity levels over a 2-year followup period (van Vugt *et al.* 2001). Another group studied less severely affected patients over 48 hours and showed increased motor activity while awake and sleeping (Hurelbrink *et al.* 2005). Actigraphy is appealing because it encompasses both motor and functional aspects of the illness; however, its utility as a marker of progression may be limited by the large stepwise changes in functional activity that occur as HD progresses.

I.2.4.5 Gait analysis

Like oculomotor function, gait disturbance is a widespread feature of HD that has traditionally been studied using expensive specialist equipment. Such studies have revealed consistent changes in several gait parameters in HD. However, in order to be useful as a biomarker, measures need to be made quickly and consistently between centres. Easy-to-use instrumented carpet devices have recently become available. Using one such device, HD patients were shown to differ significantly from controls in all parameters of gait including velocity, stride length and cadence (Rao *et al.* 2005). Such devices have not yet been tested in premanifest HD.

I.2.4.6 Graphimetry

Computerised writing analysis (graphimetry) quantifies a highly functional fine motor control task. Because no special equipment is required to perform the task, it can be performed in patients' homes and therefore may be able to provide data with high temporal resolution. Preliminary results suggest that it may be capable of identifying premanifest gene carriers who are close to developing motor features of HD (Thanendrarajan *et al.* 2007).

I.2.4.7 Tongue force

Objective measurement of tongue force has been proposed as a marker of the motor phenotype of HD that may reflect a different subset of neuropathology from other quantitative measures because motor control of the tongue may be impaired in HD when other movements are normal. Preliminary data suggest that tongue force variability correlates with severity in manifest disease (Bohlen *et al.* 2007).

I.2.5 Cognitive measures

Cognitive abnormalities in HD are well described (e.g. Craufurd *et al.* 2002). Cognitive changes have the potential to identify premanifest HD gene carriers close to developing motor signs of the disease, and are also very important in their own right. Cognitive tasks are more subject than other classes of potential biomarker to practical limitations such as difficulty in standardisation across languages, practice effects and confounding influences of psychiatric and motor symptoms of HD. Nonetheless, head-to-head longitudinal comparisons of standardised cognitive batteries may yield useful information that will not be captured by other measures. The development of such batteries forms part of the aim of several large ongoing studies, including Predict-HD (Huntington Study

Group), PHAROS (Huntington Study Group), EHDN Registry and HD-Toolkit (Euro-HD Network REGISTRY Steering Committee 2003-8; Stout 2009). Longitudinal data from these studies are awaited.

I.2.6 Discussion

The pipeline presented in Figure 14, drawn from discussions within the Euro-HD network biomarkers working group, highlights the series of steps required for a potential marker to become an approved surrogate endpoint in a clinical trial of a putative disease-modifying therapy. Biomarker candidates identified to date have considerable overlap and require head-to-head evaluation in large, longitudinal, multi-centre studies having the features of clinical trials.

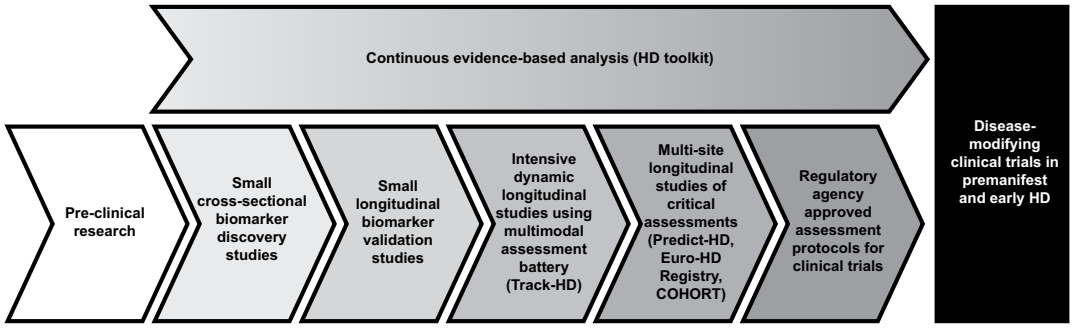

Figure 14 Pipeline for the establishment of biomarkers for HD as surrogate endpoints for clinical trials of disease-modifying therapies

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The challenges faced in establishing which biomarker candidates should be taken forward to biomarker-powered interventional trials are more than logistical. No single biomarker can be expected to encompass all aspects of HD and it is therefore likely that a combination of markers and compound measures from across all available modalities will be required to obtain a true picture of the disease process in an individual. Determining what combination of markers best captures the most significant aspects of the disease process in a sensitive and specific manner with minimal duplication is a major challenge that current and planned studies may answer only partially. Different biomarkers and combinations are likely to be required to fulfil the numerous roles for which biomarkers are needed (see Table 2), from prediction of age at onset to assessment of therapeutic efficacy.

Most importantly, perhaps, past efforts to use biomarkers as outcome measures for clinical trials demonstrate that a highly effective biomarker may behave unexpectedly when measured in concert with a putative therapeutic intervention. When whole-brain atrophy was used to assess the efficacy of beta-amyloid immunization therapy in Alzheimer's disease, the expectation was that efficacy would be signalled by a reduction in atrophy rates. Unexpectedly, treated patients experienced *more* atrophy than those given placebo, despite favourable cognitive outcomes (Fox *et al.* 2005). Whatever the explanation for this, it is clear that interactions between disease, intervention and biomarker will likely be more complex than anticipated. A biomarker is never truly *validated* but rather undergoes a constant process of *evaluation*, of which measurement in clinical trials forms part. In parallel to the pipeline presented in Figure 14, each biomarker must therefore undergo extensive investigation to establish its place in the physiological and pathological mechanisms of the HD patient so that the effects of interventions may be better anticipated.

Finally, there is unlikely to be one combination of markers that is best placed to measure the effects of every possible treatment. The intervention and biomarkers must be carefully chosen to provide both efficacy and pharmacodynamic information. For example, measures of peripheral inflammation would be pharmacodynamic if used with an anti-inflammatory drug, but may be markers of treatment efficacy if measured alongside a histone deacetylase inhibitor.

I.3 Aims of this thesis

I.3.1 Unmet needs

It is to be hoped that the preceding discussion sets out adequately both the need, and the realistic hope, for disease-modifying treatments for HD in the near future. In addition, the argument has been made for the development of biomarkers capable of tracking progression in HD, to facilitate the conduct of clinical trials of such treatments. Numerous biomarker candidates have been proposed and these now require head-to-head comparison to determine their power and redundancy. This thesis focuses on three main areas of remaining need.

First, though hypothesis-driven research has led to the identification of several candidates detectable in blood, there is a conspicuous lack of plasma proteins in the list of potential blood markers (Table 3). The use of proteomic profiling, to identify in an unbiased manner those proteins whose plasma

levels are differentially altered in HD, has not previously been reported, and little work has focused on the targeted investigation of the underlying pathological basis for differences in plasma protein expression in HD. Meanwhile, some molecules with immediate relevance to neuropathogenesis, that have been shown to track with progression in other diseases, have not been tested as possible biomarkers for HD.

Second, there are compelling reasons why a panel of biomarkers ought to include one or more volumetric imaging measures. To date, the bulk of imaging biomarker research has focused on striatal atrophy, or the examination of regional atrophy across the whole brain. Quantification of whole-brain atrophy using the robust BBSI technique has the potential to capture all pathological (and clinically-relevant) atrophy in a single measure but has only been reported as a small pilot study, and requires detailed study in a larger cohort over a longer interval, to determine both the rate and profile of whole-brain atrophy and the relationships between atrophy and existing clinical and genetic measures of severity and progression.

Finally, as set out above, the head-to-head comparison of biomarker candidates will require the establishment of longitudinal cohorts of subjects in whom a variety of measures from several biomarker modalities will need to be applied simultaneously, and compared both within and between measures using suitable statistical frameworks with sufficient power to detect significant synergy between markers while discarding redundant measures.

I.3.2 Hypothesis

- Trials of potentially disease-modifying treatments will be needed in HD in the coming years.
- The epidemiology and natural history of HD and likely number of agents requiring testing are such that enough sufficiently-powered trials will be difficult or impossible to conduct.
- Biomarkers of HD may improve the efficiency of clinical trials by reducing subject numbers and duration, and potentially increasing the validity of the outcomes *per se*.
- The existence of a sensitive and specific genetic test is capable of revealing subjects destined to develop HD and those with manifest disease: this enables completely accurate

clinical characterisation of cohorts and will facilitate the development of biomarkers despite the relative rarity of the disease.

- Existing candidate biomarkers require evaluation in larger cross-sectional and longitudinal cohorts.

- Novel techniques of analysing biofluids may reveal new biomarker candidates.

- Mechanistic evaluation of biomarker candidates revealed by such methods, in terms of pathogenic pathways of HD, may provide insights into pathogenesis, the likely behaviour of biomarkers in clinical trials, and possibly novel therapeutic approaches.

- Whole-brain atrophy measured using the BBSI has the potential to be a robust imaging biomarker of HD and requires further evaluation in a large, well-characterised cohort.

I.3.3 Aims

1. To establish and study a cohort of subjects with premanifest and early manifest HD and control subjects, and study them longitudinally over 2 years using volumetric MRI imaging, clinical and psychiatric assessment, genetic analysis and blood sample collection for biomarker development.

2. To apply unbiased proteomic discovery techniques to analyse the blood samples collected from these subjects — in concert with further blood samples collected from a wider spectrum of patients attending a Multidisciplinary HD clinic — in order to identify novel plasma protein biomarker candidates.

3. To use robust quantification techniques to evaluate biomarker candidates in terms of their reliability as markers, across disease stages cross-sectionally and with advancing disease longitudinally.

4. To use a hypothesis-driven approach to investigate biomarker candidates in terms of their mechanistic role in the pathogenic pathways of HD, with a view to establishing the reasons why they appear to behave as biomarkers, as well as shedding light on incompletely understood pathogenic pathways and possibly revealing potential targets for disease modification.

5. To evaluate the use of whole brain atrophy, quantified using the brain boundary shift integral, as an imaging biomarker in presymptomatic gene carriers and patients with early disease over the period of study

6. To use these volumetric imaging data to study anatomical correlates of salient clinical features of HD (motor and psychiatric manifestations), to determine to what extent regional atrophy measures may have promise as predictors of clinical burden.

I.4 Publications relating to this chapter

Sections of the review presented in this introductory chapter were published as:

- Wild EJ and Tabrizi SJ (2006) *Predict-HD and the future of therapeutic trials*. **Lancet Neurology** 5(9): 724-725.

- Wild EJ and Tabrizi SJ (2007) *The differential diagnosis of chorea*. **Practical Neurology** 7(6): 360-373.

- Wild EJ and Tabrizi SJ (2007) *Huntington's disease phenocopy syndromes*. **Current Opinion in Neurology** 20(6): 681-687.

- Wild EJ, Mudanohwo EE, Sweeney MG, Schneider SA, Beck J, Bhatia KP, Rossor MN, Davis MB and Tabrizi SJ (2008) *Huntington's disease phenocopies are clinically and genetically heterogeneous*. **Movement Disorders** 23(5): 716-720.

- Wild EJ and Tabrizi SJ (2008) *Biomarkers for Huntington's disease*. **Expert Opinion on Medical Diagnostics** 2(1): 47-62.

Chapter II General methods

II.1 Structure and conduct of cohort studies

THE WORK IN this thesis is drawn largely from two cohort studies, running in parallel. The *longitudinal imaging study* ran from October 2005-June 2008 and was a prospective, longitudinal study of volumetric imaging and clinical, behavioural and psychological assessment in premanifest and early HD. 81 subjects were enrolled (20 controls, 21 premanifest HD gene carriers and 40 patients with early manifest HD). This study had three major timepoints with full assessment at each: baseline, one year and two years. The year two timepoint, in practice, was delayed by three months for two reasons. Firstly, a study design with unequal assessment intervals allows each datapoint to contribute to both the slope and vertical intercept of a best-fit line. Second, a number of subjects were cross-recruited at the two-year timepoint into a complementary but larger study, TRACK-HD (TRACK-HD Steering Committee 2006-8) which, by design, used the same volumetric MRI sequence, in addition to 3T MRI imaging not reported here. Cross-recruited subjects therefore had combined visits at this timepoint, and the MRI and clinical data were shared between both studies. At recruitment, the intention was to perform a one-year, two-timepoint study but the project was extended to allow a third timepoint, and subjects were invited to extend their participation in the study by a year.

The *biofluid study* began in 2003 and is a prospective project aimed at collecting biofluid samples from controls and HD gene carriers throughout the disease course, processing and storing them under standardised conditions and performing a variety of analyses to identify and further study biomarker candidates in HD. The inclusion criteria for the study are wider in order to maximise the number of samples collected and the extent of the disease spectrum captured. The study was designed and has been run with the ethos of a biobank (Medical Research Council *et al.* 2006), with an emphasis on consistency of sample collection, processing and storage, as well as rigorous stewardship of the sample database and associated metadata. Recruitment and sample collection for this study was carried out largely through the HD Multidisciplinary Clinic of the National Hospital for Neurology and Neurosurgery, so by necessity the clinical assessments carried out were less comprehensive. The biofluid study was both cross-sectional and longitudinal, though the focus of this thesis is, for the most part, the cross-sectional findings. Subjects were invited to donate

samples of blood and urine at each clinic appointment, subject to the inclusion criteria. Longitudinal collection aimed to leave at least six months between consecutive donations. Samples of both blood and urine were collected and, while some preliminary metabolomics work has been carried out on the urine samples, this thesis only reports the findings from the study of blood.

Cross-recruitment between the two studies was encouraged. Wherever subjects were willing, subjects in the longitudinal imaging study were invited to donate a blood sample for the biofluid study, which was processed and stored using the same techniques. Samples obtained from such cross-recruitment were accompanied by reliable clinical, behavioural, cognitive and imaging data, greatly enhancing their utility in the assessment of the pathogenic relevance of biofluid biomarker candidates. In addition, a subset of subjects gave samples on multiple occasions, enabling longitudinal study of the relationships between clinical, imaging and biofluid measures.

II.2 Consent and ethical considerations

All studies mentioned in this thesis were carried out at approved research institutions. The author's own work was all carried out at University College London's Institute of Neurology in conjunction with the National Hospital for Neurology and Neurosurgery, UCL Hospitals NHS Trust.

All human experiments were carried out in accordance with the declaration of Helsinki. All human subjects gave informed, written consent to participate. Human subjects at UCL/UCLH were enrolled into either or both of two studies: UCLH/04/N008 (Identification of biomarkers that can be used to track the progression of Huntington's disease) and UCLH/03/N009 (Use of serial magnetic resonance imaging to identify outcome measures for clinical trials that may be used to track the progression of Huntington's disease (HD) – in early disease and in presymptomatic gene carriers). Both these studies were approved by University College London (UCL) / UCL Hospitals Joint Research Ethics Committee.

No animal work was carried out by the author, but this thesis includes animal work carried out by others. All animal experiments were carried out in accordance with relevant legislation and approved by local and national regulatory authorities. These are specified in the relevant methods sections for each experiment.

II.3 Subjects and inclusion criteria

II.3.1 Longitudinal imaging study

II.3.1.1 Source of volunteers

Human volunteers for the longitudinal imaging study were recruited from three sources:

- The National Hospital for Neurology and Neurosurgery (NHNN) Multidisciplinary HD Clinic research volunteer database (data held with subjects' consent);
- Direct prospective recruitment of volunteers by the author via the NHNN Multidisciplinary HD Clinic.
- The Huntington's Disease Multidisciplinary Clinic of Addenbrooke's Hospital, Cambridge. Potential subjects were referred, with their permission, by Dr Roger Barker.

Recruitment was carried out equally by the author and Dr Susie Henley, research psychologist.

II.3.1.2 Inclusion criteria

- Subjects were classified as controls, premanifest HD and manifest HD.
- For **all subjects**, the inclusion criteria were all of the following:

    1. Able to give informed consent;
    2. Age over 18 at the time of enrolment;
    3. No contraindications to MRI scanning such as metal implants, and able to tolerate the procedure, i.e. not claustrophobic;
    4. English as first language (this was necessary for reliable cognitive testing);
    5. Absence of active major psychiatric disorder (for the purpose of cognitive testing and subject retention);
    6. Absence of significant active central nervous system or medical disorders (in order to avoid confounding effects on outcome measures).

For **premanifest HD subjects** the additional inclusion criteria were**:**

7. Positive predictive genetic test for the HD CAG repeat expansion, performed by an accredited genetic testing laboratory; *and*

8. Absence of features diagnostic of clinical onset of HD, as determined by a rating of <4 points on the diagnostic confidence scale of the UHDRS (The Huntington Study Group 1996).

For **manifest HD subjects**, the additional inclusion criteria were:

9. Positive genetic test for the HD CAG repeat expansion, performed by an accredited genetic testing laboratory; *and*

10. Presence of features diagnostic of clinical onset of HD, as determined by a rating of 4 points on the diagnostic confidence scale; *and*

11. Early stage Huntington's disease, i.e. disease stage I or II according to the criteria of Shoulson and Fahn (Shoulson *et al.* 1979), as defined under the UHDRS as total functional capacity score >7.

For **control subjects** the additional inclusion criteria were:

12. Partner or spouse of premanifest or manifest HD subject (partner/spouse controls); *or*

13. Individual previously at risk of inheriting HD, with a negative genetic test for the HD CAG repeat expansion, performed by an accredited genetic testing laboratory (gene-negative controls).

II.3.1.3 Comments on inclusion criteria

The inclusion criteria were designed to enable the study of the population likely to be eligible for clinical trials of potential disease-modifying treatments in HD (see section I.1.5), while considering pragmatic factors. The main aim was to validate, in a large cohort, pilot work suggesting that whole-brain atrophy rates were increased in early HD (Henley *et al.* 2006), over a longer period in order to

extend the duration of the trial to improve signal-to-noise ratios; and to obtain preliminary data on whole-brain atrophy in premanifest HD.

It was considered that ability to complete this study — in particular, the ability of patients to attend appointments and tolerate scanning and assessment — would be influenced by a number of factors including functional ability, cognitive dysfunction, psychiatric disturbance and motor dysfunction. It was hoped that using early disease as an inclusion criterion in manifest HD would produce a cohort of subjects with a diverse range of HD features but who were at approximately the same level of functioning, and who were likely to be able to continue to attend assessments for the duration of the study.

To an extent, the population defined by these criteria is by definition unrepresentative, as features such as major psychiatric disturbance and movement disorders are both relatively common in HD, but both would diminish subjects' ability to complete the study, reducing its power. Nonetheless, these criteria allowed for a reasonable approximation of the population likely to be eligible for early clinical trials while encompassing much of the clinical diversity of the disease.

Matching across groups for gender was carried out informally at the level of recruitment, with efforts to ensure a roughly even balance of males and females in each group. Matching age and CAG repeat length across groups was more challenging, because of the interactions between CAG repeat length, age and disease duration. For a given repeat length, subjects who are younger are less likely to have disease signs. So if the premanifest and manifest groups are to be matched for CAG repeat length, the premanifest group is likely to be younger on average than the manifest group. Conversely, if age-matching is sought, the CAG repeat lengths of the groups are likely to differ significantly, since for a given age, premanifest subjects will tend to have lower CAG repeat lengths than similarly-aged subjects with manifest disease. Thus, it was decided not to attempt to match these two groups on the basis of either age or CAG repeat length, but to recruit a wide range of both ages and CAG repeat lengths, and attempt to correct for differences statistically (see section II.10). Age-matching of the control group for both groups of mutation carriers was attempted, however, by ensuring that the control group spanned the full range of ages in both groups.

Control subjects for the longitudinal imaging study were drawn from two different populations: partners and spouses, and gene-negative family members. It was felt that these individuals were likely to share with the mutation carriers a number of environmental factors that could otherwise act as confounders, such as socioeconomic status, diet, sleeping habits and living conditions. A further, significant expected advantage was that, to a degree at least, the psychological burden of living with a genetic or clinical diagnosis of HD would be common to gene carriers and their partners and spouses, thus enabling a clearer discrimination of the direct and indirect effects of the HD mutation, especially on the cognitive, behavioural and neurological measures. The same was felt likely to hold (though perhaps to a lesser extent) for gene-negative subjects, previously at risk of carrying the mutation, since many such individuals remain carers for parents and siblings with HD.

II.3.2 Biofluid study

II.3.2.1 Source of volunteers

Human volunteers for the longitudinal imaging study were recruited from two sources:

- Direct prospective recruitment of volunteers via the NHNN Multidisciplinary HD Clinic.
- Cross-recruitment of subjects from the longitudinal imaging project, to enable better phenotypic characterisation of subjects.

II.3.2.2 Inclusion criteria

- Subjects were classified as controls, premanifest HD or manifest HD.
- Manifest HD subjects were further divided into early, moderate or advanced HD.
- For **all subjects**, the inclusion criteria were all of the following:

1. Able to give informed consent;
2. Age over 18 at the time of enrolment;
3. Absence of significant active central nervous system or medical disorders (in order to avoid confounding effects on outcome measures).

For **premanifest HD subjects** the additional inclusion criteria were**:**

1. Positive predictive genetic test for the HD CAG repeat expansion, performed by an accredited genetic testing laboratory; *and*

2. Absence of features diagnostic of clinical onset of HD, as determined by a rating of <4 points on the diagnostic confidence scale of the UHDRS (The Huntington Study Group 1996).

For **manifest HD subjects**, the additional inclusion criteria were:

1. Positive genetic test for the HD CAG repeat expansion, performed by an accredited genetic testing laboratory; *and*

2. Presence of features diagnostic of clinical onset of HD, as determined by a rating of 4 points on the diagnostic confidence scale.

Manifest HD subjects were subdivided into early, moderate and advanced stages as outlined in section II.5.2.

For **control subjects** there were no additional inclusion criteria, though in practice the majority of control subjects were partners and spouses of mutation carriers.

II.4 Genetic testing

The majority of subjects in both studies underwent genetic diagnosis through the Neurogenetics Laboratory of the National Hospital for Neurology and Neurosurgery, London. However, the process of repeat length measurement has evolved in technique and improved in consistency since its introduction in 1994. Moreover, some subjects underwent clinical genetic testing in other laboratories.

Where consent could be obtained, therefore, CAG re-sizing was carried out in order to move towards a reliable standard throughout the cohorts.

CAG repeat length sizing were carried out by the Neurogenetics Laboratory of the National Hospital for Neurosurgery. DNA was extracted using standard techniques using an NA3000 automated DNA extractor (AutoGen, MA). Repeat lengths were analysed by fluorescent PCR

amplifying the triplet repeat region followed by size fractionation and fragment sizing using an Applied Biosystems 3730XL genetic analyser and GeneMapper software (Applied Biosystems, CA).

II.5 Clinical assessments

II.5.1 Longitudinal imaging study

Clinical assessment of subjects was based on that adopted by the Euro-HD Network REGISTRY study, a Europe-wide clinical database project that aims to define the phenotype of HD comprehensively (Euro-HD Network REGISTRY Steering Committee 2003-8).

Clinical assessments were as follows:

- Demographic questionnaire (Appendix A)
- Medical history questionnaire (Appendix B)
- HD history questionnaire (Appendix C)
- UHDRS motor scale (Appendix D)
- UHDRS functional scales (Appendix E)

At the baseline and one-year timepoints, these assessments were carried out by the author after training and certification as an accredited motor rater by the Euro-HD Network. At the two-year timepoint, because of cross-recruitment of subjects into the TRACK-HD study, the clinical assessments in a subset of subjects were carried out by another trained rater (Dr Nayana Lahiri).

II.5.2 Biofluid study

Because the majority of samples for the biofluid study were collected through the outpatient clinic, the clinical data collected were by necessity less comprehensive. They consisted of:

- Demographic data (Name, date of birth, gender)
- Subject group (control or HD)
- CAG repeat length (see section II.4)

- Significant comorbidities
- Medications
- Clinical stage (premanifest, early, moderate or advanced according to standard criteria defined by Total Functional Capacity (TFC) score (Shoulson *et al.*; Shoulson) and the UHDRS diagnostic confidence scale (The Huntington Study Group), as applied by trained raters (all neurologists or neurogeneticists) including the author:
  - **Premanifest HD**: UHDRS motor diagnostic confidence score < 4;
  - **Early HD**: UHDRS motor diagnostic confidence score = 4 and TFC between 13 and 7;
  - **Moderate HD**: UHDRS motor diagnostic confidence score = 4 and TFC between 6 and 3
  - **Advanced HD**: UHDRS motor diagnostic confidence score = 4 and TFC between 3 and 0
- For subjects who were cross-recruited into both the longitudinal imaging study and the biofluid study, the more comprehensive assessments in section II.5.1 were carried out, enabling examination of the relationships between more quantitative clinical and behavioural scores and biofluid findings in a subset of subjects.

II.6 Behavioural assessments

At the time of the design of the longitudinal imaging study (November 2005), the shortcomings of the UHDRS Behavioural Assessment were clear so an alternative assessment was sought. The PBA was too long to consider for this study. However, the SBA was still in draft form and had not yet been validated formally. Nonetheless, because of the Europe-wide collaborative nature of its development, that draft was widely felt to be an improvement on the UHDRS measure. The draft SBA (Appendix G) was therefore adopted as the core behavioural measure of the study. However, because it had not yet been validated it was decided that the Beck depression inventory would be employed in parallel. This was important not only to gain information about the critical affective

behavioural axis, using a well-validated scale, but also to enable the SBA to be piloted alongside the more traditional measure.

At the two-year timepoint, a slightly different, revised draft SBA was used instead, because of cross-recruitment of subjects to TRACK-HD which uses the revised form as its standard behavioural tool. The revised form differs from the first draft in several ways:

- More detailed questions about (dis)orientation in time, place and person;
- The addition of a 'worst' dimension to each domain, capturing the worst severity that it has attained over the preceding month, enabling better assessment of fluctuating symptoms;
- The addition of an irritability subscale which was absent from the first draft because of overlap with aggressive behaviour and difficulty differentiating between the two domains reliably;
- Changes to suggested prompts.

Subjects in the biofluid study did not undergo behavioural testing, unless they were cross-recruited to the longitudinal imaging study.

II.7 Magnetic resonance imaging

II.7.1 Acquisition

Subjects underwent 1.5T T1-weighted volumetric imaging on a General Electric scanner using an IR prepared FAST spoiled GRASS sequence with 24 x 18cm field of view and 256 x 256 matrix providing 124 contiguous 1.5mm-thick coronal slices. In-plane voxel dimensions: 0.9375 by 0.9375 mm. Acquisition parameters: TR = 13ms; TE = 5.2ms; flip angle = 13°; inversion time = 650ms; receiver bandwidth = 16kHz, NEX=1. The scan acquisition time was approximately 9 minutes. Subject to available time and subject agreement, two consecutive ('back-to-back') scans were obtained to maximise scan quality.

II.7.2 Pre-processing

Image processing and analysis was carried out using Medical Image Display and Analysis Software (MIDAS) (Freeborough *et al.* 1997). Automated bias correction was performed to correct for

magnetic field inhomogeneities (Boyes *et al.* 2008). This fully automated process removes some of the effects of differences in head position within the scanner on regional image intensity. Bias-corrected scans were checked for quality by a blinded expert rater. Where back-to-back scans were available, the scan with fewer artefacts was selected for analysis.

II.8 Blood sample acquisition and processing

Blood samples were collected from the antecubital fossa between 2pm and 4pm. For protein-based analyses, samples were collected into 6ml EDTA-coated Vacutainer™ tubes (BD, Oxford, UK). All samples were processed within 2 hours of collection.

The blood fractionation procedure was designed to obtain good quality plasma, as well as preserving white cell pellets for future analysis of intracellular changes. An established technique, gradient density centrifugation, was employed (Boyum 1968). Histopaque® Accuspin™ tubes (Sigma, Dorset, UK) consist of two chambers separated by a porous high-density polyethylene barrier ('frit'). The lower chamber contains Histopaque®-1077, an aqueous solution of a high molecular weight polysaccharide and sodium diatrizoate, an iodinated nonionic compound, adjusted to a density of 1.077 ± 0.001. On contact with this reagent, erythrocytes aggregate and granulocytes become slightly hypertonic, increasing their sedimentation rate, resulting in pelleting at the bottom of the tube. Lymphocytes and other mononuclear cells (including monocytes) remain at the interface between plasma and the reagent. Plasma may be collected from above this layer and the mononuclear layer may be collected separately. Contamination of plasma and the mononuclear layer by erythrocytes and granulocytes is avoided by the barrier between the chambers.

Fractionation was carried out at room temperature. Histopaque® Accuspin™-1077 tubes were centrifuged for one minute at 1000g to ensure that the reagent was confined to the lower chamber. Whole blood was added gently to the upper chamber and the tubes centrifuged for 10 minutes at 1000g. Two plasma aliquots of ~1mL were removed from above the mononuclear layer using a sterile pipette, avoiding the cell layer by 5mm. The remaining plasma, mononuclear cells and reagent were removed, leaving 5mm above the frit to minimise erythrocyte contamination. The mononuclear layer was diluted to 10mL with PBS (Sigma) and centrifuged at 250g for 10 minutes. The supernatant was decanted, resuspended to 10mL with PBS and centrifuged at 2500g for 12 minutes. The two resulting cell pellets and plasma were frozen at -70°C. Plasma aliquots were

thawed once, over ice, and divided into 100µL aliquots which were re-frozen at -70°C until required.

II.9 Cerebrospinal fluid collection, processing and storage

CSF donors were recruited through the University of British Columbia HD Medical Clinic. Gene-positive subjects were staged early or moderate according Independence Score as assessed by an experienced rater (100-80, early; 75-60, moderate) (The Huntington Study Group 1996). CSF was obtained by lumbar puncture, examined by microscopy and centrifuged to remove cells and the acellular portion was frozen at -80°C. There was no significant contamination of CSF by blood cells (median erythrocyte count 0.5×106/L, range 0-171; median leukocyte count 1.0×106/L, range 0-17). Blood samples were obtained within an hour of lumbar puncture in EDTA tubes. Plasma was extracted by 2-stage centrifugation and frozen at -80°C.

II.10 Cognitive assessment

Subjects in the longitudinal imaging study underwent, at each assessment timepoint, a battery of cognitive tests lasting about 2 hours. Details are given in Appendix H. Cognitive outcomes from these subjects are reported elsewhere and do not form a focus of this thesis. At the two-year timepoint, the majority of subjects were cross-recruited into the TRACK-HD study, which had a different cognitive battery that was incompatible with the longitudinal imaging study assessment. As a result, the subject numbers undergoing cognitive assessment at the two-year time point were considerably reduced. Subjects in the biofluid study did not routinely undergo cognitive assessment.

II.11 Statistical methods

II.11.1 Overall approach

This section sets out the statistical framework used to analyse the data contributing to this thesis, and the rationale for the approach used. The discussion here is limited to topics of relevance to both studies, or those applying to many aspects of one study. Specific analyses confined to one aspect of a single study are discussed separately in the statistical methods sections of the following chapters.

Data were collated in Microsoft® Excel 2003 (Microsoft Inc). Statistical analysis was carried out using Stata™ software versions 9 and 10 (Statacorp) and SPSS™ 14.0 (SPSS Inc).

Raw data underwent basic processing to obtain meaningful values suitable for analysis. The general statistical approach to the evaluation of each candidate measure as a possible biomarker was then based on a stepwise, cumulative analysis to determine the extent to which the measure fulfilled a number of traits:

1. Are there significant differences in the value of the measure between controls and HD patients?

2. Are there significant differences in the value of the measure between other disease groups, e.g. premanifest and manifest HD or early and moderate HD?

3. If there are sufficient numbers of clinical subgroups, is there a linear trend in the measure across all subject groups with increasing disease severity (i.e. does the measure track with progression)?

4. To what extent does the measure correlate with established measures of clinical severity?

5. To what extent does the measure correlate with what is known about the patient's risk or objective disease severity, based on their CAG repeat length and age?

6. Within the premanifest group, does the value of the measure correlate with estimated onset probability or time to predicted clinical onset?

7. To what extent is the measure capable of predicting other key characteristics of subjects, e.g. in distinguishing between controls and premanifest HD, or between premanifest and manifest disease?

8. Can the measure be combined with others to improve on the discriminating ability of the measures when applied individually?

9. Does longitudinal observation of change in the measure improve its ability to answer these questions?

II.11.2 Tests for matching between groups

Where possible, matching of groups for key demographic features was carried out at the level of recruitment. However, because of the progressive nature of HD and the relationships between CAG repeat length, age and disease duration, as well as limitations in the available pool of volunteers, differences between disease groups were almost inevitable (see section II.3.1.3). Tests were therefore performed to establish whether there were statistically significant differences between groups for the following key variables:

- Age (two-tailed t-test)
- Sex (Fisher's exact test)
- CAG repeat length between disease groups (two-tailed t-test)

For longitudinal data, inter-group differences in the inter-visit interval were also tested using a two-tailed t-test. Baseline IQ was tested between groups for the analysis of cognitive data, but not for other outcomes.

II.11.3 Covariates

The presence of a statistically significant difference in a potential confounding variable between groups is an important indicator of the need to correct for such differences in any subsequent analysis. However, the absence of such a statistically significant difference when groups are compared directly does not rule out the possibility that the variable may still exert a significant effect on other analyses. For example, a group of premanifest and early HD patients may differ slightly but not statistically significantly in terms of age, but if there is a large effect of age on brain atrophy rate, this might produce a spurious positive result when atrophy rates are compared between groups.

Thus, it is necessary to identify in advance those variables that might theoretically be expected to have a systematic effect on the value of measures of interest. Linear regression analysis allows these 'nuisance' variables to be adjusted for statistically. In addition, where required (e.g. for graphical display), the linear regression model can be used to generate values for the variable of interest, adjusted for the effect of the nuisance variable. In effect, this standardises each value to what it would be if the value of the nuisance variable were equal throughout the subject group: for

instance, using this technique to adjust atrophy rates for age produces individual values for what each subject's atrophy rate would be if all subjects were the same age. This model allows each covariate to have a different effect on the outcome measure but does rely on the assumption that the relationship between each nuisance covariate and the outcome measure is linear.

In general, age and gender were used as covariates in all linear regression analyses, while IQ was added when cognitive variables were analysed. The use of age is clear-cut, as most biological processes are affected to some extent by age, and age differences between groups were expected.

Deciding whether to adjust all analyses, or some, for a possible effect of gender is less straightforward. On the one hand, there is no firm evidence to suspect that the pathogenesis or clinical progression of HD differs between males and females (Marder *et al.* 2000; Bates *et al.* 2002) and, of relevance to the longitudinal imaging study, there are no gender-related differences in brain atrophy due to normal ageing (Good *et al.* 2001) or in neurodegenerative disease (Schott *et al.* 2005). In addition, each covariate added to a linear regression model increases the degrees of freedom which increases the size of resulting confidence intervals and increases the chance that a true effect will be missed. On the other hand, gender does exert diverse effects and could be theorised *a priori* to influence most possible marker candidates in HD. In addition, there are certain features of HD that do appear to be gender-specific such as endocrine phenotype (Markianos *et al.* 2005; Markianos *et al.* 2007) and the increased propensity of CAG repeats to expand during gametogenesis (Pearson 2003), which might conceivably be recapitulated in the somatic tissues. Overall it was felt that gender effects were unlikely but that a gender effect was sufficiently plausible that gender was used as an additional covariate in the linear regression analyses.

II.11.4 Tests for normality of data

Linear regression modelling and other parametric statistics rely (among other requirements) on the data in question having a normal (Gaussian) distribution. Formal statistical testing for whether data are normally distributed can be performed, but such tests can fail to detect non-normality in small data sets and in larger data sets may falsely infer that a small or localised deviation from the normal distribution is 'not significant'. Instead, visual comparison of the distribution of the data was used to establish normality or otherwise, by inspection of simple histograms and comparisons of data

quantiles against those of a normal distribution with equivalent parameters. Examples of such plots are given in Figure 15.

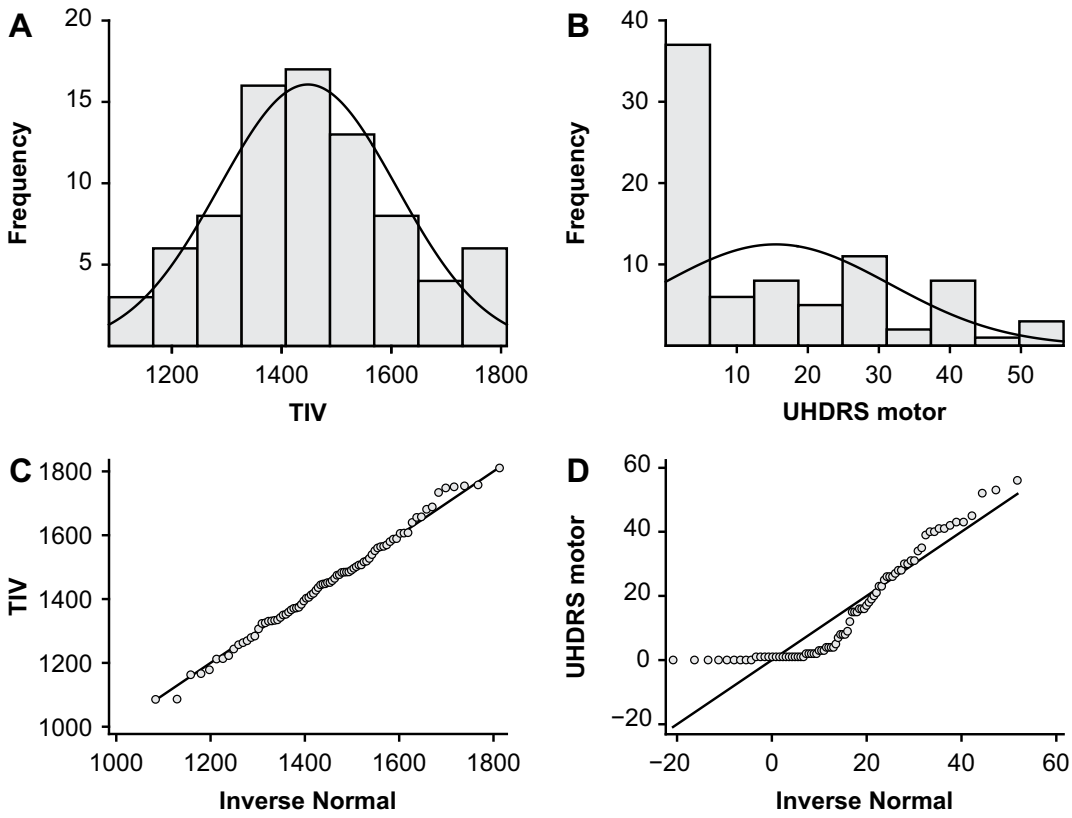


Figure 15 Examples of normal and non-normal data from the longitudinal imaging study

*(**A**-**B**) histograms with normal overlay and (**C**-**D**) inverse normal quantile plots for (**A**, **C**) total intracranial volume (TIV) and (**B**, **D**) UHDRS motor score at the baseline of the longitudinal imaging study. Inspection of such plots was used to determine whether assumptions of normal distribution were warranted (as in the case of TIV) or not (as in the case of UHDRS motor score, which is skewed towards lower scores but also shown by the normal quantile plot to fit poorly throughout the range of scores). In a normal quantile plot, a hypothetical normal distribution is calculated, having the same mean and variance as the sample data. For each sample data point, the quantile of that point is used to calculate the value of the corresponding quantile of the normal distribution. The two quantiles are plotted against each other and compared with a line of unity. Rather than showing raw quantile values, the horizontal axis is labelled with 'inverse normal' values, back-calculated from the normal distribution quantiles into the original units of measurement, so that each axis is labelled with values meaningful for the data set under comparison.*

II.11.5 Handling of non-normal data

Three options were available for the statistical analysis of data that were not normally distributed. Non-parametric tests of hypothesis can be used but these are more likely to produce false negative results, cannot be used with covariates to adjust for the effect of nuisance variables and cannot be compared directly with the results of equivalent parametric tests. Simple mathematical adjustment of data can be attempted to transform the data into a normal distribution (e.g. log-transformation) but such transformation is not always possible. Finally, the data can be resampled using a bootstrapping technique. This involves repeatedly constructing a new sample set based on random resampling a subset of subjects within a sample to produce a new data set; some subjects may be sampled more than once and others may be omitted. This is repeated many times (typically 2-3000). It enables the use of the same parametric statistical models as were applied to normally distributed data, including covariates, without having to apply specific mathematical transformations. Bootstrapping was therefore used for the analysis of all non-normally distributed data. 3000 repetitions were employed, with resampling on a per-group basis where appropriate, and confidence intervals were calculated with bias-correction (Thompson *et al.* 2000).

II.11.6 Correction for multiple comparisons

Over the two parallel projects contributing to this thesis, a large number of statistical comparisons was performed. The main contributor to the multiplicity of tests was the analysis of associations between candidate biomarkers and the numerous clinical measures (see section II.11.9) in which each potential marker was tested against each clinical measure, using up to three models per pairing. Performing a large number of statistical tests brings with it a risk of false-positive results. Opinion among statisticians is divided on whether or not to make Bonferroni-type corrections for multiplicity in analyses of this type, with some authors insisting that they should always be employed (e.g. Bender *et al.* 2001) but a number (e.g. Rothman 1990; Savitz *et al.* 1995; e.g. Perneger 1998; Thompson 1998) arguing against their use. Overall, the approach adopted in this thesis was in keeping with the overall consensus that they should only be routinely used when the associations being investigated are so 'similar' that they are not of independent scientific interest. Thus, it was necessary to use multiplicity correction when performing inter-group comparisons between the different clinical stages in the biofluid study (section II.11.7) because adjacent clinical stages differ in only a quantitative manner and there is overlap between groups; but since the battery of clinical

and cognitive tasks was designed to minimise redundancy while ensuring a reasonably comprehensive phenotypic coverage, multiplicity correction was not deemed necessary in general. For a few notable exceptions where there is considerable overlap in assessments (e.g. UHDRS total functional capacity and independence score), the number of tests was reduced by excluding the less useful measure from the analysis (in this cohort, IS). The use of proteomic discovery methodology (Chapter III) inevitably brings with it a necessity for multiple comparison testing and this is discussed separately in that chapter.

II.11.7 Inter-group comparisons

Inter-group comparisons of the mean value of candidate markers were used to address the first two general statistical questions (section II.11.1):

- Are there significant differences in the value of the measure between controls and HD patients?

- Are there significant differences in the value of the measure between other disease groups, e.g. premanifest and manifest HD or early and moderate HD?

Where there were two or three groups of interest (as in the longitudinal imaging study) a linear regression model was used; when used to test for significant differences between pairs of groups, this model has the properties of a t-test but allows the use of covariates. Where there were more than two groups (as in the biofluid study), the number of inter-group statistical tests required was of concern. One option might have been to reduce the number of tests and only, say, test between adjacent groups (for a four-group study, this would reduce the number of tests from nine to three). However, it was felt that statistically differences between non-adjacent groups would also be of interest, so an alternative strategy was adopted. One-way analysis of variance (ANOVA) was used to determine whether the mean value in one or more groups differed significantly from that in the other groups. Where the ANOVA indicated that one or more means was sufficiently different, post-hoc Tukey HSD ('honestly significant difference') tests were then employed to test for significant differences between all pairs of groups. The Tukey HSD test is a pairwise parametric mean-comparison tests that is more-stringent than a t-test because it incorporates correction for multiple

comparisons (Kutner 2005). Where the ANOVA indicated that no group's mean was significantly different from the others, no post-hoc tests were performed.

II.11.8 Assessment of trends across groups

Assessment of trends across groups aims to address the question:

- If there are sufficient numbers of clinical subgroups, is there a linear trend in the measure across all subject groups with increasing disease severity (i.e. does the measure track with progression)?

Testing whether a measure tracks with progression is a key facet of biomarker evaluation because markers that differ significantly between groups but do not proceed linearly with advancing disease (e.g. measures whose value rises early but then falls later) are more difficult to model and use as surrogate endpoints than those that track linearly. The inter-group tests above, while providing important information about individual groups and their relationships, do not make use of the full information available from the design of the study. A single test that could encode the known fact that, overall, disease severity increases in each group from premanifest to early to moderate HD would be desirable. In addition the control group may effectively be considered a model for gene carriers with no exposure to mutant huntingtin.

Linear regression analysis was therefore used to construct a model for the four-group design of the biofluid study. Group membership was encoded by assigning integer values to each group: controls, 0; premanifest HD, 1; early HD, 2; and moderate HD, 4. Each variable of interest was then regressed against this group code, with age and sex as covariates. Because the group code values are arbitrary, the constant term of this analysis is not interpretable, but the partial correlation coefficient R can be used to quantify the goodness-of-fit of the pattern of the variable across all groups and, since the arbitrary group codes are the same for each analysis, it can be compared directly across groups. Like the constant term, the slope of the association is not meaningful in itself but the p-value for whether it differs significantly from zero is a valid measure of the significance level of the across-group linear trend.

This approach was used to construct figures comparing the values of potential markers across groups in the biofluid study. It was felt that with three-groups or fewer, this test of linearity would be

susceptible to undue influence by one group, so this analysis was not performed for the longitudinal imaging study.

II.11.9 Associations with clinical measures

This analysis addresses the question:

- To what extent does the measure correlate with established measures of clinical severity?

Clear mechanistic associations with disease pathogenesis are a highly desirable feature of a candidate biomarker (see discussion in chapter I.2.6) and in their absence it may be impossible to predict how a biomarker may perform in concert with a putative disease-modifying treatment. Brain tissue cannot readily be obtained *in vivo* from human HD patients, so mechanistic connections must be explored indirectly. As discussed in I.2, existing clinical tools have substantial shortcomings but are the current 'gold standard' for clinical trials and many of them do, on a population level, show strong associations with both the severity of neuropathology and 'hard' clinical outcomes such as disability and death (Vonsattel *et al.* 1985; The Huntington Study Group 1996). Therefore it is desirable to establish whether the pattern of a candidate biomarker correlates well or poorly with other clinical measures. A strong correlation in the expected direction is relatively easy to interpret, as it suggests that the biomarker captures the disease process to a similar extent to the clinical marker. However, owing to the absence of a true 'gold standard' in HD weaker correlations, or those in a counter-intuitive direction, are harder to explain, as discrepancies may be due to one of several factors: a shortcoming of the new measure; a shortcoming of the traditional clinical measure; or simply that each measure is capturing a different but potentially important facet of the disease process. The latter two are the most dangerous pitfalls because they may lead to rejection of a valuable and genuine biomarker on the basis of a poor degree of association with an existing, and imperfect, clinical measure. Thus, clinical correlations alone cannot be used alone to establish the pathological relevance or clinical applicability of a potential biomarker, but do certainly contribute to the overall picture that ultimately establishes a marker's utility over time.

Clinical correlations with key clinical, psychiatric and cognitive variables were assessed using linear regression analysis, using age and sex as covariates, in the premanifest and manifest groups. Unlike the scores discussed above determined from age and triplet repeat length, most clinical scores are

expected to show different distributions between premanifest and manifest disease. A stepwise approach, from most to least stringent, was adopted to modelling the possible interactions, as outlined in Figure 16. The most complex model allowed for the association between the candidate marker and the clinical measure to have a different slope (and intercept) in each group. This model was adopted if the term for the interaction between group and the clinical measure was significantly different from zero at the $p<0.05$ level. If it was not, a model was examined in which the slopes of the associations did not differ, but the groups were allowed to have different intercepts, i.e. the value of the candidate marker was significantly different between groups for a given value of the clinical measure. This model was adopted if the term for the difference between groups was significantly different from zero at the $p<0.05$ level. If it was not, a final model was fitted in which the association between the candidate marker and clinical measure had the same slope across both groups, which were also not allowed to differ from each other in terms of absolute value. If the slope term was not significantly different from zero at the $p<0.05$ level, the model was rejected and no correlation was deemed to exist.

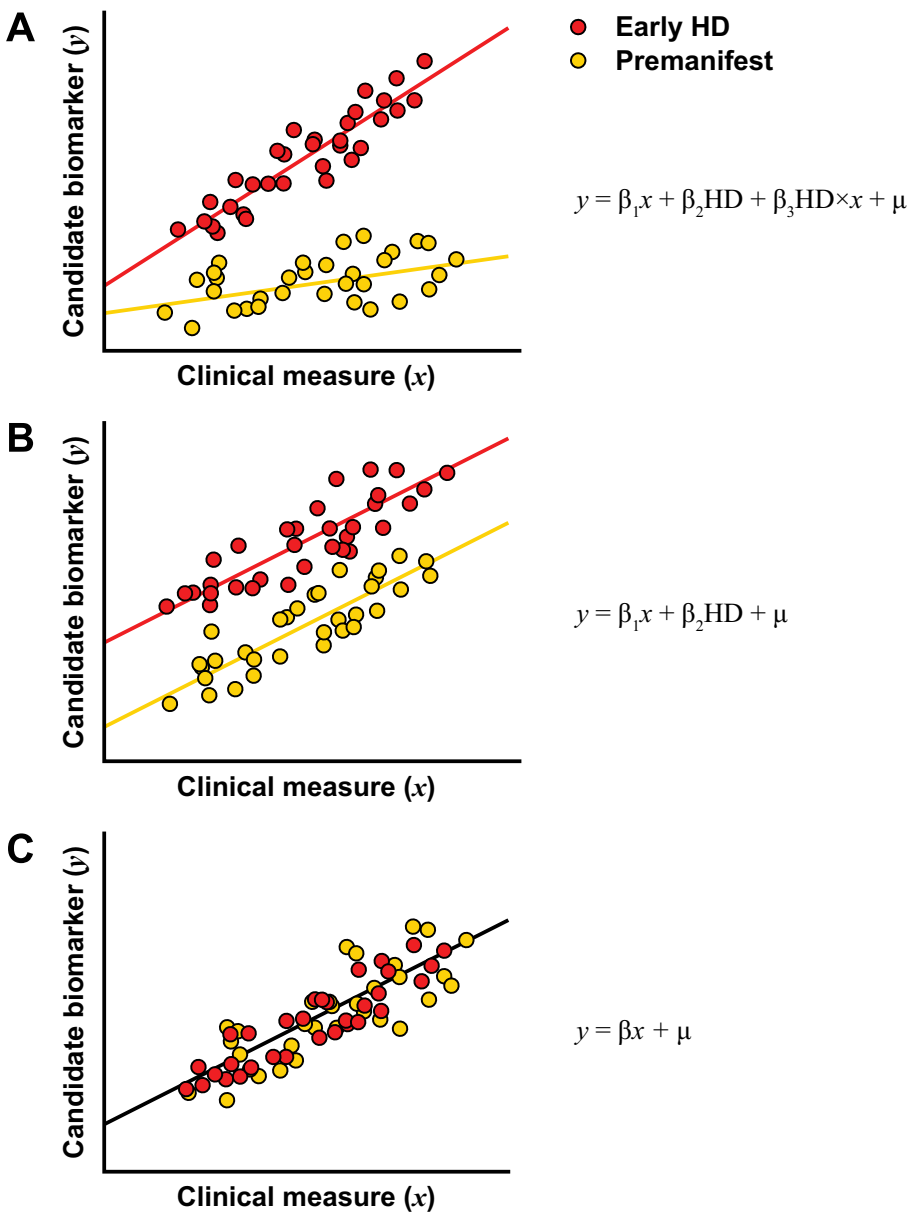


Figure 16 Linear regression models sequentially applied to clinical correlation analysis

*In the most complex model (**A**), the slope of the correlation between the candidate biomarker value (**y**) and the clinical measure (**x**) is different in the premanifest and manifest groups if the* **group×x** *interaction term (β_3) is significantly different from zero. In the next model (**B**), the slope of the interaction is not allowed to differ between groups but the values are significantly different between groups overall, if the group term (β_2) is significantly different from zero. In the final model (**C**), the association between* **x** *and* **y** *has the same slope and the two groups do not differ in value from each other.*

II.11.10 Modelling genetic predictors of disease course

As discussed in chapter I.1.1.2, there is no established measure of disease severity that can be used to place both premanifest gene carriers and patients with overt disease onto a single scale — a 'universal x-axis' — to determine to what extent a putative biomarker tracks linearly with the progression of neuropathology. One approach is to use age on its own as the comparator, and certainly as age increases, so does the burden of pathology, but of course at a given age, different subjects will have not only different clinical phenotypes but also very different degrees of pathology. CAG repeat length is the only factor shown to have a consistent and quantifiable influence on both the age of motor onset and severity of pathology (e.g. Duyao *et al.* 1993; Penney *et al.* 1997; e.g. Li *et al.* 1998) and, though it certainly does not account for the full range of phenotypic variability, in the absence of a direct measure of pathological severity, a calculation taking into account age and CAG repeat length could theoretically produce an estimate of an individual's lifetime exposure to mutant huntingtin toxicity, with which the most promising putative markers would be expected to correlate. Such measures are necessary to address the question:

- To what extent does the measure correlate with what is known about the patient's risk or objective disease severity, based on their CAG repeat length and age?

One such measure is the model of disease burden proposed by Penney and colleagues (Penney *et al.* 1997), who studied the relationship between CAG repeat length and the degree of striatal atrophy post-mortem in HD brains. They found that the severity of atrophy was a linear function of CAG repeat length and age at death and that the CAG repeat length at which no atrophy was predicted to occur was 35.5 repeats. From this they proposed the formula:

$$striatal\ dysfunction = constant \times age \times (CAG - 35.5)$$

Based on the authors' inferences that "the rate of development of striatal pathology should solely be a function of the CAG repeat number" and that "striatal pathology should develop linearly from birth" this formula has since been adapted for generic use (Sanchez-Pernaute *et al.* 1999) as a theoretical measure of disease burden (with arbitrary units) in the form:

$$disease\ burden = age \times (CAG - 35.5)$$

There are a number of limitations of this approach, as has been pointed out by others (Rosenblatt *et al.* 1998). First, it is derived from cross-sectional post-mortem data, with its inevitable bias towards end-stage brain changes (which can only be avoided by limiting study to subjects who have died prematurely from causes unrelated to HD). The validity of using such data to make inferences about the progression of pathology *in vivo* is limited. Second, the study of Penney *et al.* was based on quantification of caudate atrophy, which, based on MRI measurements, is certainly selectively increased early in the disease course but does not appear to increase linearly throughout the disease as Penney and colleagues propose (Aylward *et al.* 2004), nor is there any reason to believe that the trajectory of caudate atrophy is capable of acting *per se* as a universal marker of pathology.

A more widely-accepted measure of the combined contributions of age and CAG is the conditional onset probability calculation of Langbehn and colleagues (Langbehn *et al.* 2004). Based on a very large cohort of 2913 individuals from 9 countries, it uses a parametric survival model to predict for a given subject the probability p that they will remain disease-free for a specified number of years (conventionally five):

$$p = 1 - \left(\frac{1 + e^{\frac{\pi}{\sqrt{3}}} \times \frac{e^{9.56 - 0.146 \times CAG} + age_{now} - 21.54}{\sqrt{35.55 + e^{17.71 - 0.327 \times CAG}}}}{1 + e^{\frac{\pi}{\sqrt{3}}} \times \frac{e^{9.56 - 0.146 \times CAG} + age_{onset} - 21.54}{\sqrt{35.55 + e^{17.71 - 0.327 \times CAG}}}} \right)$$

Where age_{now} is the subject's current age, age_{onset} is the projected disease-free age (e.g. age_{now} + 5) and CAG is the subject's CAG repeat length.

This can be rearranged to predict, for a given individual, the number of years of disease-free life that must elapse before they reach a conditional onset probability of interest (conventionally 50 or 60%):

$$years\,to\,onset = \frac{\sqrt{3}}{\pi} \times \ln\left(\frac{1 + e^{\frac{\pi}{\sqrt{3}} \times \frac{-21.54 - e^{9.56 - 0.146 \times CAG} + age_{now}}{\sqrt{35.55 + e^{17.72 - 0.327 \times CAG}}}}}{1 - p} - 1 \right) \times \sqrt{35.55 + e^{17.72 - 0.327 \times CAG}} + 21.54 + e^{9.56 - 0.146 \times CAG} - age_{now}$$

Where age_{now} is the subject's current age, CAG is the subject's CAG repeat length and p is the conditional onset probability of interest.

Conditional onset probabilities calculated using this method have several advantages. First, they are based on genuine population data from a large sample using the most widely agreed-upon event in premanifest HD: diagnosis of motor onset. Second, they make no assumptions of linearity of progression but, rather, attempt to model the non-linearity of onset probability. Third, unlike models that simply use an individual's CAG repeat length to predict their probability of onset at a given age, this **conditional** model makes use of the additional clinical information that the individual has survived to age_{now} without developing motor signs, to modify the onset probability. For example, an individual with a repeat length of 44 would be predicted, at birth, to have a 77% probability of onset by the age of 50 years; but if the same individual remained disease-free at 45, their *conditional* probability of onset by the age of 50 would be reduced to 53%, because of the information about their genotype-phenotype concordance encoded in 45 years' disease-free survival.

Because of these advantages, the Langbehn *et al.* model is widely used in the study of premanifest gene carriers (e.g. Paulsen *et al.* 2006). It was therefore used as a standard comparator in the analysis of biomarker candidates discussed in this thesis, for the premanifest cohort. However, though the modelling of the interaction between CAG and age is considerably more sophisticated than in the Penney *et al.* formula, because the model was developed in premanifest subjects, and moreover carries the assumption of a lack of motor signs at age_{now}, it would be meaningless if applied to subjects with manifest disease and cannot be used to carry out analyses across both groups.

Nonetheless such analyses are certainly of interest, and the approach of Penney *et al.* was adapted to construct a linear regression model suitable for comparing across premanifest and manifest HD. Variables of interest were regressed against CAG repeat length, using age as a covariate, in premanifest and early HD. One way to interpret this model is that is asks what the effect of CAG would be on the value of the outcome measure, if all subjects had the same age; that is, what is the additional effect on the measure of each additional CAG triplet for a subject with a given age. This model makes two assumptions. First, it assumes that the effect of CAG repeat is linear. This is

certainly not true throughout the range of pathological CAG repeats, as the model of Langbehn et al shows; but it is a reasonable assumption within the range of repeat lengths to be expected from the studies' inclusion criteria (section II.3.1.2). Second, it assumes that the effect of CAG is equal in premanifest and manifest HD — that is, there is nothing about having disease signs *per se* that influences the effect of CAG on the outcome measure. This assumption can be sidestepped by introducing an interaction term for an effect of CAG × group, but given that the aim of the model is to examine the pattern of the measure across all gene carriers, agnostic to disease status, this analysis was not attempted.

II.11.11 Discriminatory ability of biomarker candidates and marker combinations

One test of the utility of a biomarker is the degree to which it can be used in isolation to predict known phenotypic features of subjects that can be measured by other means. Assessing this aims to address the question:

- To what extent is the measure, alone or in combination, capable of predicting other key characteristics of subjects?

This analysis is complementary to that of associations between marker candidates and clinical measures (section II.11.9) and we would expect those measures that performed well in the latter to do so in the former. In a sense, it is a more demanding trial of the potential biomarker, since rather than making a statement about the strength of a given association, and whether that association is statistically significant, it tests the ability of the candidate marker to select the more likely of two choices that the investigator has determined to be of relevance, modelling some of the real-life functions biomarkers would be expected to fulfil, such as distinguishing between control subjects and premanifest gene carriers, or between premanifest and manifest HD.

The approach adopted for this task was logistic regression analysis, which is used fairly routinely in medicine in the evaluation of potential biomarkers and their ability to assist in clinical and diagnostic decision-making (e.g. Yuen *et al.* 2007), and has been applied in the field of neurodegeneration (Teunissen *et al.* 2003). It is a form of generalised linear model in which the distribution of a variable in a sample is used to predict a dichotomous outcome. The contrasts tested were those key clinical outcomes for which outcomes could be determined reliably for each subject in these cohorts:

- Controls vs. premanifest HD carriers (testing whether the candidate marker can distinguish subjects that clinical assessments cannot);

- Premanifest HD vs. manifest HD (testing the ability of the marker to diagnose motor onset - see discussion in chapter I.1.1.2);

- Controls vs. all HD gene carriers (testing whether the marker can detect a generic signal present in all mutation carriers).

In addition, it is of considerable interest to be able to analyse markers not only alone but in combination and it is likely that combining biomarkers in this way will be necessary to provide the power and breadth of scope necessary for the conduct of future clinical trials (see chapter I.2.6). The key question is

- Can the measure be combined with others to improve on the discriminating ability of each measure applied individually?

Where multiple measures are available on a single subject on given occasion, *stepwise* logistic regression can be used to test them systematically. Briefly, this is an algorithmic approach to the selection of significant predictors of an outcome a logistic regression analysis is performed on each variable of interest, and those variables that significantly improve the prediction the outcome are included in the model, which is re-tested after the addition of each 'significant' variable. Thus a final model is arrived at which combines the best predictive power of the variables of interest, giving an idea of the 'added value' of each measure over the others (Hanley *et al.* 1982). Stepwise logistic regression was therefore used to compare measures of interest within the multivariate biofluid study, and also to compare across modalities in analysing the discriminatory ability of combinations of biofluid and imaging markers. The use of stepwise logistic regression is not without its pitfalls and the method has been criticised for being too lenient, and performing multiple tests on the same data (Copas 1983). However, with judicious selection of the variables of interest and careful interpretation of the data, the technique can be a valuable way of assessing the real-life utility of markers and examining the possible value of combining them.

A stepwise logistic regression model can also be used to construct receiver operating characteristic (ROC) curves, a standard graphical representation of the characteristics of a clinical test with a dichotomous outcome. By convention, the results of such a test can be classified in four ways: true positives and true negatives (the test correctly assigns the subject to the 'positive' or 'negative' outcome; and false positives and false negatives (the test incorrectly assigns the subject). A ROC curve plots the true positive against the false positive diagnosis rate, such that for each subject whose status is determined by the test to be 'positive', a correct result causes the plotted line to deviate upwards, while an incorrect result causes it to deviate to the right. A test that assigned subjects randomly to either outcome would tend to produce a diagonal line, while a perfect test would produce a vertical line. In practice, most tests fall between the two extremes and it follows that the area under the curve (AUC) is one measure of the ability of the measure to classify cases correctly (i.e. the discrimination of the test): the closer the AUC is to 1, the better the discrimination. Indeed a system of classifying tests exists, according to their the AUC values they produce when ROC curves are plotted (Hanley *et al.* 1982):

Area under the curve (AUC)	Discrimination of the test
More than 0.9	Excellent
0.8 - 0.9	Good
0.7 - 0.8	Fair
0.6 - 0.7	Poor
Less than 0.6	Fail

Table 5 Classification of test discrimination according to the area under the ROC curve

II.11.12 Sample size estimation

For several measures of interest, it was desirable to calculate estimates of sample size requirements for clinical trials using modification of that measure as an outcome. In addition to providing useful information in themselves, samples size estimates have been published in the literature, or can be calculated from it, for some existing candidate biomarkers in HD (e.g. Aylward *et al.* 2003).

Calculations of the sample size that would be required for an observational study to detect a simple difference in a measure between controls and a patient group were calculated using the formula:

$$n = \frac{2(z_{\alpha/2} + z_{\beta})^2(\sigma_1 + \sigma_2)^2}{(\mu_2 - \mu_1)^2}$$

Where:

n is the estimated sample size required per group

α is the desired significance level (typically 0.95 for 'p<0.05' results)

$1\text{-}\beta$ is the desired power (typically 0.9 for 90% power). Power is the probability of identifying a correlation if one exists

z_p is the ordinate for the normal distribution for a given probability p

σ_1 is the standard deviation of the measure in the control group

σ_2 is the standard deviation of the measure in the patient group

μ_1 is the mean value of the measure of the control group

μ_2 is the mean value of the measure of the patient group

The calculation of sample size requirements for interventional clinical trials is more complex and depends on the manner in which we wish to model the effect of the intervention. The effect size of the treatment, E, is a value between zero and one that expresses the proportion by which the treatment is expected to reduce the change in the measure. The key decision rests on whether the effect of normal variability in controls is to be included in the model. If we omit control data from the model, the treatment is predicted to reduce the absolute value of the measure by E towards zero, and the sample size is given by:

$$n = \frac{(z_{\alpha/2} + z_{\beta})^2 {\sigma_2}^2}{E^2 {\mu_2}^2}$$

For example, in the case of brain atrophy, with an effect size of 0.3, this would model a 30% absolute reduction in atrophy rate (including any atrophy due to normal ageing). However, if control data are included in the model, the treatment is modelled as having an effect of magnitude E on the **difference** between patients and controls for the measure and the sample size requirement is given by:

$$n = \frac{(z_{\alpha/2} + z_{\beta})^2 (\sigma_1 + \sigma_2)^2}{E^2 (\mu_2 - \mu_1)^2}$$

For the example of brain atrophy, this would mean reducing by 30% the **excess** atrophy caused by the disease process, above normal ageing.

The difference is not trivial, because the sample size requirements generated by the two approaches can vary greatly. For instance, data from the pilot study of whole-brain atrophy in early HD (Henley *et al.* 2006) produce requirements of 268 subjects per arm if control atrophy is included, and 156 if it is excluded, for a 6-month trial of an intervention with 30% effect, at 90% power and 5% significance. The organisation of clinical trials will require a single reliable figure for funding, planning and recruitment. On the face of it, the latter (relative reduction) approach seems more biologically plausible, because no intervention would realistically be expected to act equally on a pathological process in patients and the ageing process in normal controls: in the example given it is unlikely a treatment would halt age-related brain atrophy. However, values for outcome measures obtained from observational trials already include a contribution from normal variability and ageing, by definition. Moreover, normal subjects are not generally included in clinical trials, because both trial arms typically consist of patients, one under active treatment and the other receiving a placebo, so the additional inclusion of normal variability appears less clearly warranted. In this thesis, sample size calculations are based on the absolute reduction in atrophy, excluding control data, but conservative effect sizes have been used, because the treatment would be expected to attain a smaller reduction in an absolute measure.

Subject drop-out from longitudinal studies can also be taken into account for sample size estimations (e.g. Fox *et al.* 2000) and is usually estimated at 10% per year.

II.11.13 Analysis of longitudinal data

Both studies contributing to this thesis involve the longitudinal collection of data or samples from the same subjects over multiple timepoints. For measures that can be calculated cross-sectionally (e.g. most biofluid measures), the expectation is that longitudinal observation may reveal a stronger disease-related signal, because of the expectation that, within each subject group, the change in that measure over time is likely to be less variable than its baseline value, if the disease exerts a

differential, progressive effect on the biological processes underlying the measure that is greater in HD gene carriers than in controls (see Figure 10).

II.11.13.1 Longitudinal data with two timepoints

The majority of measures arising from the parallel studies were cross-sectional, but where serial measurement of such cross-sectional measures was performed, longitudinal rates of change were calculated by subtracting the baseline value from that at followup and, where necessary, dividing by the time interval between observations to produce an annualised rate of change:

$$rate\,of\ change = \frac{followup - baseline}{interval}$$

In calculating longitudinal change rates, one issue is whether the change score should be analysed as an absolute value or adjusted for the baseline value. A ten-point change in a given score over one year may have a different meaning depending on whether the baseline score was ten or ten thousand. On the other hand, given that the aim of measuring change longitudinally is to seek to eliminate some of the heterogeneity seen cross-sectionally, it may defeat the object to adjust for baseline score, especially if the cross-sectional values are highly variable within groups but do not differ greatly between groups.

If adjustment for baseline values is to be attempted, different approaches can be considered. The first is to calculate the change value as a proportion of the baseline value, *viz*:

$$rate\,of\ change = \frac{followup - baseline}{baseline} \div interval$$

Alternatively, the change score can be analysed in its unadjusted form, using the baseline value as a covariate.

Both of these approaches to adjustment for baseline value have a potential shortcoming in that a proportional adjustment may amplify or diminish change scores. Consider two subjects observed at two timepoints over one year. Subject A's score halves from 200 to 100 while subject B's score doubles from 200 to 400. The absolute change scores would be -100 and +200 respectively, while the proportional changes would be -50% and +100%. But if the nature of the change is such that it

acts directly on the baseline value — as in the case of brain atrophy, where the process of atrophy directly diminishes brain volume — a halving and doubling of volume could be seen as equivalent changes in different directions. Expressing the change logarithmically as

$$change, \delta = \ln\left(\frac{followup}{baseline}\right) \div interval$$

gives values of -0.69 and +0.69 respectively, capturing the perceived equivalence of these changes.

In practice, brain atrophy was the only outcome for which the outcome measure was felt to be a sufficiently immediate reflection of the process under observation, acting directly on the entity measured at baseline, to warrant such correction. Followup brain volume was calculated by subtracting the absolute volume change measured by the BBSI from the baseline brain volume. After analysis, logarithmic change rates were back-converted to meaningful atrophy rates using the formula

$$annualised\ atrophy\ rate = \left(1 - e^{\delta}\right) \times 100$$

For other measures in the longitudinal imaging study, absolute change scores were used for the analysis.

Once an appropriate annualised change rate was calculated, it was treated as a new measure and analysed as set out in sections II.11.2-II.11.11. For the analysis of associations with clinical measures, it was felt most useful to examine correlations between pairs of longitudinal measures, rather than examining, say, associations between baseline clinical score and longitudinal change in a candidate marker.

II.11.13.2 Linear change over more than two timepoints

The longitudinal imaging study had three timepoints, meaning that up to three values were available for all cross-sectional measures, with the caveats noted in section II.5. The longest interval would be expected to have the greatest signal-to-noise ratio, and therefore one approach would be to ignore the intermediate timepoint and calculate rates of change over this interval. However, given the asymmetry of inter-assessment interval, the likelihood that dropout will diminish subject numbers at

each successive timepoint, and the fact that each point value may be considered equally reliable, it is desirable to explore methods of modelling linear change that are capable of including multiple timepoints per subject in calculating rates of change. The method adopted was the generalised estimating equation (GEE), which is an extension of the generalised linear model that allows multiple observations per subject to be analysed against time to produce estimates and confidence intervals for rates of change (Liang *et al.* 1986). One feature of the GEE is that it accounts for the likelihood that subject dropout may be influenced the variable under scrutiny (i.e. to depend on disease severity), which would otherwise tend to bias the calculation towards lesser rates of change.

For a three-timepoint study, the GEE uses three sequential cross-sectional values to calculate a rate of change. BBSI-derived atrophy rates, however, are calculated directly by analysing the change in a variable and are said to be a 'direct measure of change'. Instead of three sequential values, the BBSI calculated over a three-timepoint study yields three non-sequential atrophy rates, between timepoints 1 and 2, timepoints 2 and 3, and timepoints 1 and 3. Because these do not correspond to sequential point values, the GEE cannot be used to combine all the available data to infer overall rates of change from such 'direct' measures. Some authors have successfully applied a linear mixed model to account for this difficulty (Frost *et al.* 2004). Overall, however, it was felt that a key difference between direct and indirect measures of change was the precision of each measure. This is expected to be equal at each timepoint for serially-applied point measures such as clinical scores; but a direct measure calculated over one year is likely to be less precise than the same measure applied over two years. Therefore for the direct measure of BBSI-derived whole-brain atrophy, linear analysis was restricted to calculation of change over the longest measured interval for each subject.

II.11.13.3 Linearity of change

For the three-timepoint longitudinal imaging study, comparison of change over the first and second one-year intervals allowed an assessment of whether change in each measure was linear. Annualised change in each measure over the second interval was compared with that over the first by means of groupwise paired t-tests. For groupwise comparisons of linearity, an acceleration value, calculated by subtracting the first year's annualised change score from the second, was compared between groups using linear regression, with age and sex as covariates, as described in section II.11.7. Clinical and genetic factors predicting non-linearity were assessed as described in sections

II.11.9 and II.11.10. For whole-brain atrophy, the first and second year's directly measured atrophy values were used to in place of derived change scores for paired t-tests and to calculate the acceleration value.

Chapter III Inflammatory activation in HD revealed by proteomic discovery

III.1 Introduction

THE PATHOLOGICAL HALLMARK of HD is striatal neuronal cell loss but there are widespread changes in the CNS (Bates *et al.* 2002) and systemic abnormalities have been identified including endocrine dysfunction (Björkqvist *et al.* 2006) and immune activation (Leblhuber *et al.* 1998). Peripheral abnormalities are of significance in their own right but may also reflect central pathology and, where implicated molecules cross the blood-brain barrier, may exert central effects on brain pathogenesis.

In addition, because of its slow clinical progression and the limitations of standard clinical rating scales (The Huntington Study Group 1996), there is a need for biomarkers of onset and progression in HD to power clinical trials of potential disease-modifying treatments (Henley *et al.* 2005; Handley *et al.* 2006). Unlike in other degenerative diseases, diagnostic markers are not required because of the reliable genetic test for HD (Henley *et al.* 2005). Several candidate biomarkers in plasma have been proposed (Borovecki *et al.* 2005; Hersch *et al.* 2006) but no plasma proteins to date have been reported to be effective biomarkers for HD. Because of its ready accessibility, plasma is an appealing site for biomarker discovery and evaluation, while cerebrospinal fluid, while difficult to obtain, offers closer insights into the CNS milieu.

Proteomic profiling has previously been applied to mouse models of HD and human brain tissue (Zabel *et al.* 2002) but has not been reported for human plasma or CSF. Using a multi-platform proteomic profiling approach with 2 different depletion techniques and protein identification methods, 18 candidate proteins were identified that were differentially expressed in human plasma at various stages of HD including pre-manifest gene carriers. Candidate proteins were assessed mechanistically in terms of known roles in the pathogenesis of HD and other neurodegenerative diseases. The most promising protein in this respect, clusterin, was evaluated further by immunoblotting and enzyme-linked immunosorbent assay (ELISA) quantification in plasma samples from 2 independent populations, as well as in matched CSF and plasma samples. To investigate the neuroinflammatory processes highlighted by the proteomic discovery, interleukin-6 (IL-6) levels were investigated in both human plasma and the R6/2 transgenic mouse model of HD using ELISA.

III.2 Contributions and collaborations

Collection and processing of matched CSF and plasma was performed by B Leavitt. 2D gel electrophoresis and corresponding statistical analysis was performed by A Dalrymple and R Joubert of Proteome Sciences Inc. Quantitative immunoblotting and human clusterin ELISA were performed by A Dalrymple. The Mouse IL-6 ELISA was performed by M Björkqvist of Lund University. Animal rearing and IL-6 ELISA was performed by K Sathasivam and G Bates of King's College London. Subject recruitment and clinical characterisation, plasma processing, statistical analysis of immunoblotting and ELISA experiments, preparation of figures and drafting of the published manuscript were performed by the author. Study, design and interpretation and revision of the manuscript was shared between all collaborators including the author.

III.3 Subjects and methods

III.3.1 Ethical approval and subject recruitment

Ethical approval and subject recruitment were as specified in Chapter II.

III.3.2 Inclusion and exclusion criteria

Subjects with concomitant CNS disorders, significant medical comorbidity, known liver dysfunction, recent alcohol or substance abuse, and those taking medications or supplements suspected or known to interfere with the experimental methods used, were excluded. In view of the likelihood of nutritional, infective and inflammatory disorders in advanced HD, such patients were not included in validation experiments.

III.3.3 Collection and fractionation of blood samples

Subjects were classified as controls, premanifest HD or early, moderate or advanced HD. Sample collection, fractionation and storage and CAG repeat sizing were carried out as outlined in Chapter II. Subjects' clinical data are shown in Table 6.

Experiment	Disease stage	Number of subjects	Female: male	Mean age (range)
Overall subject pool	Control	74	(43:31)	44 (21-74)
	Premanifest	42	(24:18)	40 (27-61)
	Early	58	(32:26)	46 (29-67)
	Moderate	66	(38:28)	51 (23-77)
	Advanced	10	(2:8)	54 (40-68)
2D electrophoresis 1st plasma study	Control	10	(8:2)	49 (29-67)
	HD patients	10[a]	(7:3)	51 (31-70)
2D electrophoresis 2nd plasma study	Control	55	(28:27)	43 (22-68)
	Premanifest	14	(7:7)	37 (30-46)
	Early	15	(9:6)	43 (29-51)
	Moderate	15	(9:6)	48 (24-62)
	Advanced	10	(2:8)	54 (40-68)
Immunoblotting Plasma	Control	15	(6:9)	47 (24-68)
	Premanifest	15	(7:8)	38 (30-46)
	Early	15	(9:6)	43 (29-51)
	Moderate	15	(9:6)	48 (24-62)
Clusterin ELISA Plasma	Control	18	(13:5)	42 (21-74)
	Premanifest	18	(12:6)	41 (27-61)
	Early	19	(10:9)	48 (29-63)
	Moderate	18	(13:5)	55 (35-77)
Clusterin ELISA Matched CSF and plasma	Control	9	(6:3)	45 (25-66)
	Early	9	(4:5)	51 (38-64)
	Moderate	11	(1:10)	53 (38-72)
IL-6 ELISA Plasma	Control	34	(19:15)	41 (21-67)
	Premanifest	16	(8:8)	38 (30-61)
	Early	23	(14:9)	44 (29-67)
	Moderate	23	(13:10)	50 (23-69)

Table 6 Clinical characteristics of subjects in each study for discovery and evaluation experiments

[a]*4 early, 4 moderate, 2 advanced HD patients. Independent sample sets were used for the two discovery experiments and CSF/plasma pairs; other plasma work was performed using a combination of overlapping and unique samples.*

III.3.4 Collection and fractionation of CSF samples

CSF was collected, processed and stored as detailed in chapter II.9. 20 subjects with HD and 10 control subjects, age-matched and lacking the HD mutation, were recruited (see Table 6).

III.3.5 2D gel electrophoresis

Two independent 2D gel electrophoresis (2DE) studies were performed using different depletion and identification techniques to maximize sensitivity. For the first study (minimal depletion), plasma samples from 10 HD patients and 10 controls (Table 2) were depleted of albumin and immunoglobulins using an antibody-based spin column (GE healthcare, UK). For the second study (stringent depletion), plasma samples from 55 HD patients and 55 matched controls (Table 2) were depleted of the 6 most abundant proteins using Multiple Affinity Removal System (MARS) HPLC columns (Agilent Technologies, CA).

Depleted protein pellets were re-suspended in 2D gel lysis buffer composed of 9.5 M urea, 2% CHAPS (USB Corporation, OH), 1% dithiothreitol, 0.8% Pharmalyte (pH 3-10, GE healthcare) and Mini Complete Protease Inhibitor Cocktail (Roche, UK). The protein concentration of each plasma sample was determined using the Bradford assay (Bio-Rad, UK). Plasma samples (75 or 100µg in first and second study respectively) were loaded onto immobilized pH 3-10 non-linear gradient strips then separated in the second dimension using SDS polyacrylamide gels as described (Heinke *et al.* 1999; Weekes *et al.* 1999).

Resultant 2DE gels were silver-stained with OWL silver stain (Insight Biotechnologies, UK) and image analysis performed using ProgenesisTM Workstation, (version 2003.02, Nonlinear Dynamics, UK). Images were processed using the automatic wizard for spot detection, warping and matching, followed by manual editing and optimal matching to the reference gel (>80% per gel). Following background subtraction and normalization to total spot volume, spot data from the first study were analyzed using student t-tests at the 95% confidence level. Spot data from the second study were imported into StatistiXL software (www.statistixl.com) and the mean of normalized volume, coefficient of variation, fold change and Mann-Whitney test determined (pairwise comparisons between groups). A program developed internally was used to select statistically significant spots automatically based on the following criteria: spots present within at least 60% of all gels (across all subject groups), $\geq$1.5-fold change and $p<0.005$.

Significant spots from the first study were excised, prepared and analyzed by LC/MS/MS as previously described (Standen *et al.* 2003). Proteins present in the spots were determined using Mascot search algorithm against the NCBI non-redundant (www.ncbi.nlm.nih.gov) and Swiss-Prot (expasy.org) databases. Significant spots from the second study were excised, prepared and analyzed using matrix-assisted laser desorption/ionisation-time of flight (MALDI-ToF) mass spectrometry as previously described (Joubert *et al.* 2001). Mass spectra were matched with the Ms-Fit program (prospector.ucsf.edu) and the Swiss-Prot database was used to identify proteins.

III.3.6 Evaluation of clusterin as a biomarker

III.3.6.1 Semi-quantitative immunoblotting

Based on the results of the first 2DE experiment, the availability of quality commercial ELISA kits and the identification in the literature of plausible mechanistic roles in HD pathogenesis for the proteins, both clusterin and beta-actin were selected for further analysis. The concentrations of α and β-clusterin and β-actin were determined by semi-quantitative immunoblotting in 60 plasma samples (Table 6). Control experiments using a range of plasma dilutions and primary antibody dilutions were performed to ensure that quantification was within the linear range. For α and β-clusterin, undepleted plasma samples (2µg) were used; for β-actin, plasma samples were depleted using MARS HPLC columns and 20µg used. Plasma was denatured in Laemmli sample buffer and size-separated using 12% tris-glycine gels (National Diagnostics, UK). Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (GE Healthcare). Transfer efficiency and equal loading of protein samples was assessed by incubating membranes with Ponceau red solution (Sigma) and gels post-transfer with EZblue solution (Sigma). Membranes were washed with PBS-T and incubated with blocking buffer, then with anti-α-clusterin (1:10000, 05-354, Upstate, UK), anti-β-clusterin (1:40000, sc-6419, Santa Cruz Biotechnology, CA) or anti-β-actin (1:250, A5316, Sigma) antibodies, followed by horseradish peroxidase-conjugated sheep anti-mouse (α-clusterin and β-actin, GE healthcare) or rabbit anti-goat secondary antibodies (β-clusterin, Jackson laboratories, ME). For protein band visualization and quantification, membranes were incubated with ECL-plus, scanned and analyzed using ImageQuant software (GE healthcare). Linear regression analysis with coded variables for each subject group (control=1, premanifest=2, early=3, moderate=4) was used to identify significant trends with advancing disease. Data were re-analyzed using age as a covariate

to account for any effect of age on clusterin concentration. Comparisons between individual subject groups were made using ANOVA with post-hoc Tukey HSD analysis.

III.3.6.2 Human clusterin ELISA

Clusterin concentrations in paired CSF and plasma samples (n=29, Table 1) and in a larger set of plasma samples (n=73, Table 1) were measured using a commercially available ELISA kit (RD194034200, BioVendor, Modrice, Czech Republic; manufacturer's protocol). Absorbances were determined at 450nm. Clusterin concentrations were determined from standard curves. Statistical analysis was performed as described for immunoblotting experiments.

III.3.7 Exploration of the acute phase response in HD

III.3.7.1 Human IL-6 ELISA

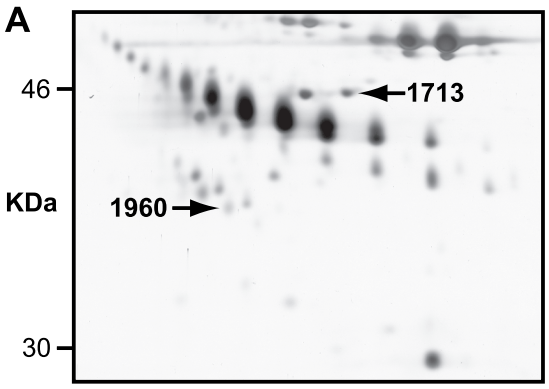
Plasma IL-6 concentrations in 96 samples (Table 6) were determined using IL-6 ELISA kits (EK-033-31, Phoenix Pharmaceuticals, Belmont, CA; manufacturer's protocol). Absorbance and concentration were determined and statistical analysis performed as for clusterin ELISA.

III.3.7.2 Mouse IL-6 ELISA

Affected mice were hemizygote R6/2 females (Induced Mutant Resource, Bar Harbor, ME), bred and reared by backcrossing R6/2 males to (CBA × C57BL/6) F1 females (Harlan Olac, UK). Transgenic animals were identified by PCR of tail-tip DNA. CAG repeat size determination (Woodman *et al.* 2007), housing conditions and environmental enrichment (Hockly *et al.* 2003) were as previously described. Animals had unlimited access to rodent breeding chow from a food hopper. Mice were subject to a 12h-light, 12h-dark cycle. Animals were culled by cervical dislocation and blood was withdrawn. After clotting, serum was obtained by centrifugation and snap-frozen. 50µL aliquots were used for the IL-6 ELISA (M6000B, R&D systems, UK; manufacturer's protocol). Absorbance and concentration were determined and statistical analysis performed as described for clusterin ELISA.

III.4 Results

III.4.1 2D gel electrophoresis



B

Spot number	Protein or proteins in spot	Fold change	*p* value (t-test)
1713 (2 proteins)	β-actin Apolipoprotein A-IV precursor	↑7.3	0.00007
1960	Clusterin precursor (α and β chains)	↑2.0	0.004

Figure 17 Proteins identified by 2DE and LC/MS/MS

A. *Representative 2D gel region depicting spots 1713 and 1960 that were significantly upregulated in 10 HD patients compared with 10 controls.* ***B***. *Proteins identified by LC/MS/MS. Spot 1713 contained 2 proteins, β-actin and ApoA-IV; spot 1960 contained the α and β chains of clusterin. (Data supplied by A Dalrymple and R Joubert.)*

1273 unique protein spots were analysed in total. In the first 2DE study, 2 spots (termed 1713 and 1960) were significantly upregulated in HD patients compared with control plasma samples (Figure 17A). LC/MS/MS confirmed that spot 1713 contained β-actin and ApoA-IV (7.3-fold change compared with controls; p=0.00007) and spot 1960 contained the α and β chains of clusterin (2-fold change compared with controls; p=0.004) (Figure 17A and B). Whilst the LC/MS/MS data for spot 1960 provided direct evidence for glycosylation on the individual clusterin chains, it was not possible to elucidate the exact nature of the post-translational modification due to the limited sequence coverage. Several isoforms of clusterin were observed in the 2DE experiment: hence spot 1960 represented a fraction of the total clusterin population. A representative control gel is given in Figure 18.

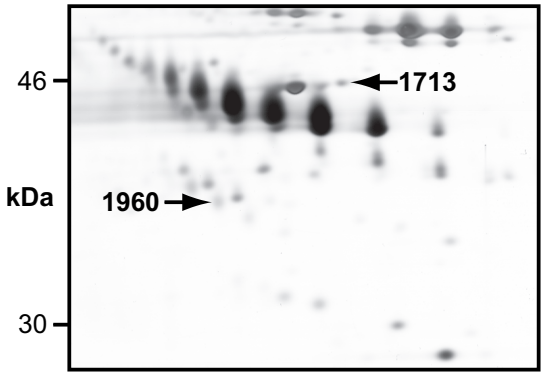


Figure 18 Representative control 2D gel region

Gel depicts spots 1713 and 1960 that were significantly upregulated in 10 HD patients compared with 10 controls. C, Control; P, premanifest HD; E, Early HD; M, Moderate HD. (Data supplied by A Dalrymple and R Joubert.)

The second 2DE study detected an additional 15 spots that were significantly different between control and HD plasma (Table 7). These included complement components C7 and C9, alpha-2 macroglobulin and alpha-2 antiplasmin (all significantly upregulated in HD versus controls, Table 7A); and afamin, insulin-like growth factor binding protein and plasma retinol binding protein (all significantly downregulated in HD versus controls, Table 7B). Increasing levels of clusterin, β-actin and ApoA-IV with advancing disease were not observed by 2DE following MARS column depletion: this was assumed to be an effect of the depletion as the first 2DE study was performed on less stringently depleted samples. However, the same sample cohort was used for immunoblotting, where clusterin is demonstrated to increase with disease progression (Figure 2).

A. Proteins increased with progressing HD clinical stage.

Spot no.	Protein or proteins in spot	Observation	Fold change	*p* value (t-test)	p_{adj}
151	Complement component C7 precursor	C < P	3.36	0.005	0.1
	Alpha-2-macroglobulin precursor				
357	Alpha-2-antiplasmin	E < M	1.58	0.004	0.1
376	Complement component C9	C < A	1.61	0.0004	0.002
		P < A	1.99	0.0001	0.002
		E < A	1.72	0.0001	0.002
		M < A	1.51	0.0003	0.007
578	Fibrinogen gamma chain	E < A	1.55	0.01	0.2
660	Haptoglobin precursor	C < A	2.58	0.003	0.07
		E < A	2.47	0.009	0.2
661	Haptoglobin precursor	C < A	3.77	0.006	0.1
		P < A	2.56	0.02	0.5
		E < A	2.78	0.03	0.7
		M < A	3.42	0.006	0.1
725	Complement C4 precursor	C < P	2.97	0.02	0.5
789	Haptoglobin precursor	C < A	3.87	0.009	0.2
		E < A	4.68	0.006	0.1
		M < A	3.19	0.01	0.2

B. Proteins decreased with progressing HD clinical stage.

Spot no.	Protein or proteins in spot	Observation	Fold change	*p* value (t-test)	p_{adj}
259	Afamin	C > A	1.86	0.005	0.1
		P > A	2.08	0.0001	0.002
		E > A	1.80	0.004	0.1
283	Inter-alpha-trypsin inhibitor heavy chain H4	P > A	2.29	0.0009	0.02
	Prothrombin		2.19	0.003	0.07
		E > A			
291	Insulin-like growth factor-binding protein complex acid labile chain	E > A	3.25	0.0006	0.01
	Prothrombin	E > A			
297	Inter-alpha-trypsin inhibitor heavy chain H4	P > A	2.26	0.0009	0.02
608	Leucine-rich alpha-2-glycoprotein	P > A	4.31	0.004	0.1
614	Serum paraoxonase/arylesterase 1	P > A	1.63	0.0004	0.01
699	Clusterin	E > A	1.95	0.002	0.05
703	Inter-alpha-trypsin inhibitor heavy chain H4	E > A	3.44	0.004	0.1
783	Plasma retinol-binding protein precursor	C > A	1.82	0.0004	0.01
		E > A	1.60	0.003	0.07
		M > A	1.51	0.003	0.07

Table 7 Proteins identified by 2DE and MALDI-ToF to be regulated in HD patients and controls

C, control; P, premanifest; E, early; M, moderate; A, advanced. P_{corr}, Bonferroni-corrected p-values adjusted for multiple inter-group comparisons (24 per protein). (Data supplied by A Dalrymple and R Joubert.)

III.4.2 Semi-quantitative immunoblotting

Representative post-transfer gels are shown in Figure 19.

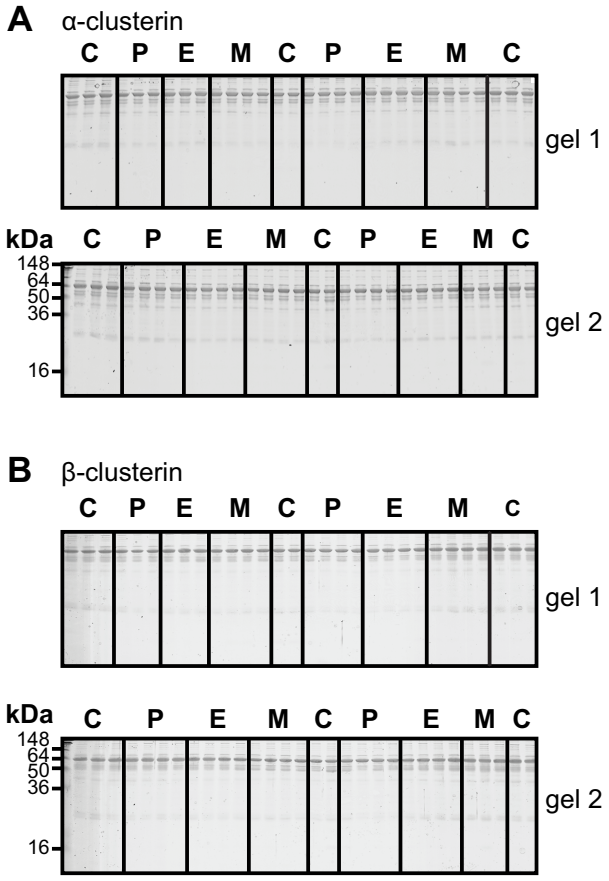


Figure 19 Post-transfer SDS-PAGE gels demonstrating equal protein loading of samples

*Gels are shown for (**A**) α-clusterin and (**B**) β-clusterin. Gels were stained with EZblue solution (Sigma) to determine protein loading. C, Control; P, premanifest HD; E, Early HD; M, Moderate HD. (Data supplied by A Dalrymple.)*

Semi-quantitative immunoblotting demonstrated increased levels of both α- and β-clusterin with HD progression (Figure 20). The trend for rising levels with increasing disease severity across all groups was statistically significant for both α-clusterin (p=0.000003) and β-clusterin (p=0.0007). These trends remained significant when allowing for any effect of age and sex on clusterin concentration in the regression analysis (p=0.0001 and p=0.0002 respectively). There were also significant differences between individual groups (ANOVA with post-hoc Tukey HSD analysis). For α-clusterin: early>control (p=0.0052); moderate>control (p=0.0001); moderate>premanifest (p=0.0073). For β-

clusterin: moderate>control (p=0.0082). β-actin expression did not track with disease progression (data not shown).

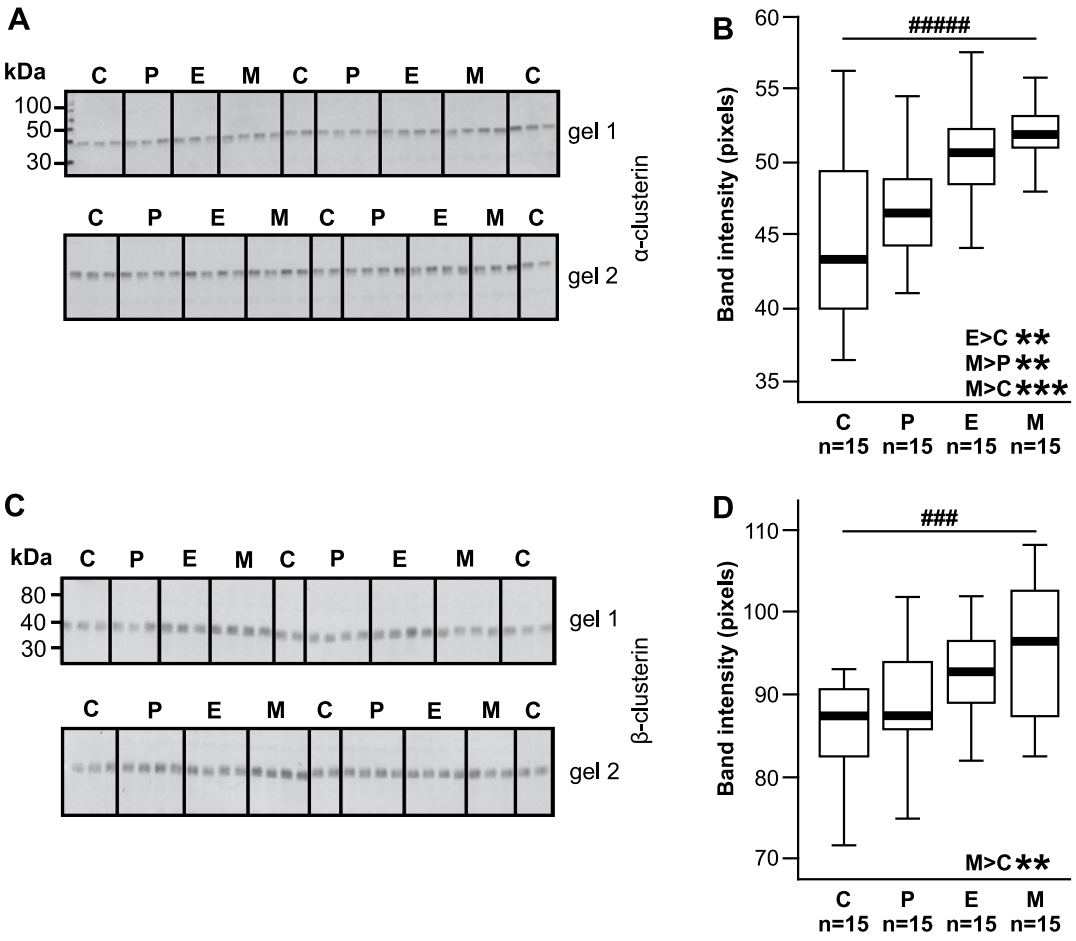


Figure 20 Semi-quantitative immunoblotting studies to determine α- and β-clusterin expression

*Representative western blots and box-and-whisker plots of band intensity values for α-clusterin (**A** and **B**) and β-clusterin (**C** and **D**). Samples were applied in pseudorandom order to avoid systematic error across gels. Mean values ± SD for α-clusterin: control (C) 44.9 ± 5.72; premanifest (P) 46.8 ± 3.47; early HD (E) 50.3 ± 4.14; moderate HD (M) 52.0 ± 3.16. Mean values ± SD for β-clusterin: C 86.2 ± 5.79; P 90.0 ± 6.84; E 93.2 ± 7.75; M 95.1 ± 8.68. The overall trend for increasing clusterin across all groups, using linear regression analysis, was significant for both α- and β-clusterin (#####p=0.000003 and ###p=0.0007 respectively). Significant differences between individual groups are shown (ANOVA with post-hoc Tukey HSD test: **p<0.01; ***p<0.001). (Raw data supplied by A Dalrymple.)*

III.4.3 Clusterin ELISA

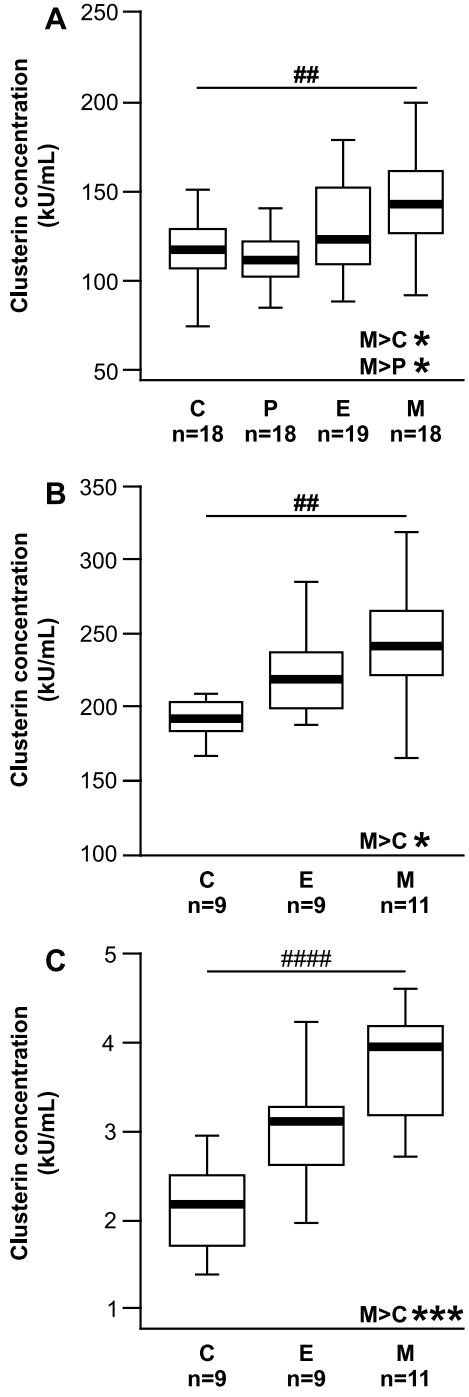


Figure 21 Quantitative analysis of clusterin concentrations in HD plasma and CSF using ELISA

***A**. Box-and-whisker plot showing clusterin concentrations in plasma samples from UK subjects. Mean concentrations ± SD: control (C) 121 ± 25.6; premanifest HD (P) 120 ± 29.2; early HD (E) 129 ± 26.7; moderate HD (M) 149 ± 32.2. **B**. Box-and-whisker plot showing clusterin concentrations in plasma*

samples from Canadian subjects. Mean concentrations ± SD: C 192 ± 30.8; E 223 ± 30.0; M 248 ± 52.4. ***C****. Box-and-whisker plot showing clusterin concentrations in matched CSF samples from Canadian subjects. Mean concentrations ± SD: C 1.50 ± 0.7; E 2.02 ± 0.5; M 2.64 ± 0.5. The overall trend for increasing clusterin across all groups, using linear regression analysis, was statistically significant in all three studies (##p=0.0032 for UK plasma, ##p=0.0036 for Canadian plasma and ####p=0.00009 for Canadian CSF). Significant differences between individual groups are shown (ANOVA with post-hoc Tukey HSD test: *p<0.05; ***p<0.001). (Raw data supplied by A Dalrymple.)*

Reliability of the clusterin ELISA assay was demonstrated by its low mean coefficient of variation (7.1%).

ELISA experiments confirmed increased clusterin expression with HD stage in samples from both patient populations studied and in both plasma and CSF (Figure 21). Statistically significant increases across subject groups from control through progressing disease were found in the plasma from UK subjects (p=0.0032) and plasma and matched CSF from Canadian subjects (p=0.0036 and p=0.00009 respectively). These trends all remained significant when allowing for any effect of age and sex on clusterin concentration in the regression analysis (p=0.0146, p=0.0352 and p=0.00003 respectively); clusterin concentration correlated only very weakly with age (R^2=0.0429). There were also significant differences between individual groups (ANOVA with post-hoc Tukey HSD analysis). For UK plasma: moderate>control (p=0.0251); moderate>premanifest (p=0.0147). For Canadian plasma: moderate>control (p=0.0147). For CSF: moderate>control (p=0.0003).

The apparent lower plasma clusterin concentration overall in UK plasma compared with Canadian plasma is likely due to the different blood fractionation techniques employed in the two populations.

III.4.4 IL-6 ELISA

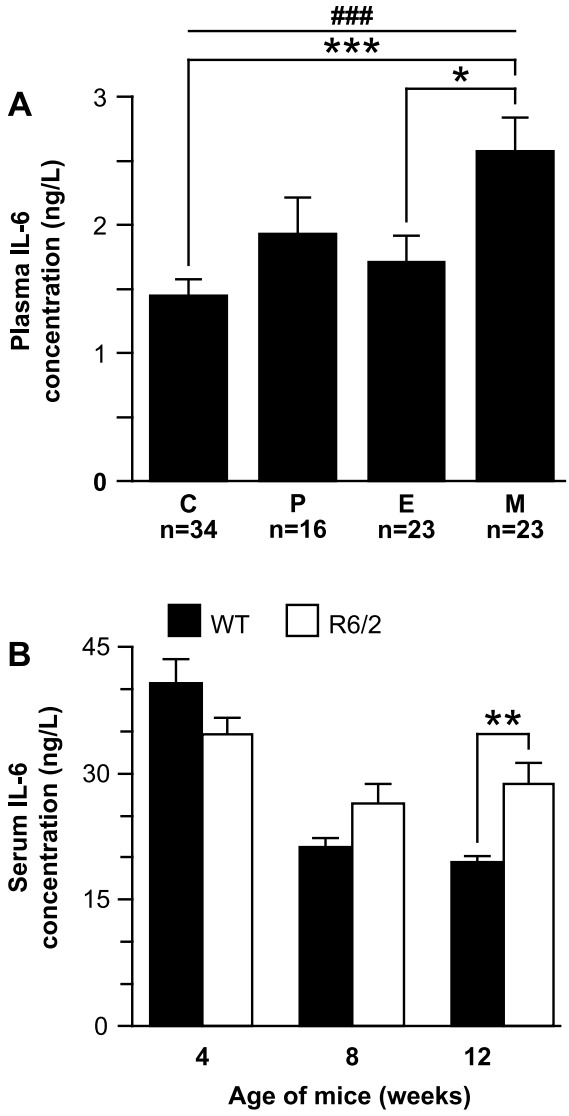


Figure 22 IL-6 ELISA quantification in HD patients and R6/2 mice

A. *Plasma IL-6 concentration (ng/L) with standard error bars in controls and HD patients. Mean concentrations ± SD: control (C) 1.45 ± 0.733; premanifest HD (P) 1.93 ± 1.12; early HD (E) 1.71 ± 1.01; moderate HD (M) 2.89 ± 1.98. The overall trend for increasing IL-6 across all groups, using linear regression analysis, was statistically significant (###p=0.0004). Significant differences between individual groups are shown (ANOVA with post-hoc Tukey HSD test: *p<0.05; ***p<0.001).* ***B***. *Serum IL-6 concentration (ng/L) with standard error bars from wild-type (WT) and R6/2 mice. n=15 for both groups at 4 weeks, 15 WT and 13 R6/2 at 8 weeks and 15 WT and 17 R6/2 at 12 weeks. Serum IL-6*

*was significantly elevated in R6/2 compared with controls at 12 weeks (**p=0.0013, t-test). (Raw data supplied by M Björkqvist and G Bates.)*

ELISA quantification of IL-6 in human plasma demonstrated a statistically significant trend to increase across advancing disease groups (p=0.0004) as well as significantly increased IL-6 in moderate HD compared with both controls and premanifest HD (Figure 22A). This trend remained significant when allowing for any effect of age and sex on IL-6 concentration in the regression analysis (p=0.0023); IL-6 concentration correlated only very weakly with age (R^2=0.0700). There were also significant differences between individual groups (ANOVA with post-hoc Tukey HSD analysis): moderate>control (p=0.0004); moderate>premanifest (p=0.0227).

ELISA data from R6/2 HD transgenic mice confirmed these findings, with mean serum IL-6 concentration significantly higher in R6/2 than wild-type at 12 weeks (p=0.0013) (Figure 22B).

III.5 Discussion

III.5.1 Immune activation in HD identified by proteomic discovery

While the proteomic discovery experiments revealed several candidate biomarkers, it should be noted that 2-dimensional gel electrophoresis has innate limitations in exploring the proteome. A eukaryotic cell is estimated to contain 50,000 proteins. The ~1300 protein spots identified here therefore represent a small fraction (~3%) of the complete proteome. 2DE is typically less successful in capturing proteins with low abundance, high or low mass, alkaline pH, hydrophobic properties or membrane localisation (Beranova-Giorgianni 2003). While positive findings from 2DE discovery are informative, therefore, useful information cannot be inferred from proteins not detected by the technique.

Several of the 18 proteins identified by our 2 proteomic discovery techniques in human plasma that track with disease progression are involved in regulation of the innate immune system, which has been reported as activated in HD (Leblhuber *et al.* 1998).

Alpha-2-macroglobulin (A2M) is an acute-phase protein, whose release is stimulated by IL-6 (Han 1997). C7 and C9 are components of membrane attack complex (MAC), whose formation is modulated by clusterin (Choi-Miura *et al.* 1996). Clusterin, which was also shown to track with

disease progression by semi-quantitative immunoblotting and ELISA in plasma and CSF, is found in almost all mammalian tissues and biofluids but is differentially expressed by certain cell types, and tissue-specific isoforms exist. Its expression is upregulated in a variety of physiological and pathological states including apoptosis and response to injury. It is implicated in diverse mechanisms of cytoprotection, membrane recycling and regulation of membrane attack complex formation. A unified role has been proposed as a heat-shock or chaperone protein with cytoprotective properties (Jones *et al.* 2002).

Our IL-6 ELISA findings in human plasma and the R6/2 mouse confirm, and may demonstrate a unifying trigger for, the presence of innate immune activation in HD. IL-6 is a pro-inflammatory cytokine that induces the release of acute-phase proteins including alpha-2-macroglobulin (Han 1997). The acute-phase response leads to the activation of the complement cascade via C3 and hence the production of downstream factors (such as C7 and C9) and modulating factors such as clusterin (Han 1997).

This peripheral immune activation may have important consequences. Inflammatory factors have profound physiological effects that may explain features of the HD phenotype. Il-6, for instance is involved in the regulation of energy balance by decreasing food intake and increasing energy expenditure, possibly through its action on the hypothalamus (Cancello *et al.* 2004). Il-6 also triggers corticosteroid release by acting on the hypothalamic/pituitary/adrenal axis (Perlstein *et al.* 1991), which may explain the derangement of the hypothalamic-pituitary-adrenal axis observed in R6/2 mice and humans (Björkqvist *et al.* 2006). Together, these effects may contribute to such phenotypic phenomena as the unexplained weight loss observed in HD (Sanberg *et al.* 1981).

III.5.2 Neuroinflammation and HD pathogenesis

Molecules produced by peripheral tissues as a result of disease processes may cross the blood-brain barrier and exert effects on the CNS. Neuroinflammation likely occurs as a result of locally and systemically produced factors. Complement activation and production of C7 have been demonstrated by microglia in the striatum of HD patients (Gasque *et al.* 2000). The classical complement pathway is activated by amyloid-β (Aβ) components; amyloid and complement proteins (including the MAC) colocalize in Alzheimer's disease (AD) brain (Gasque *et al.* 2000); and MAC components introduced into the brain of live rats induce neurodegeneration (Xiong *et al.*

2003). Apoptotic cells bind complement, activating the classical pathway, suggesting a possible mechanism of complement activation and bystander damage in HD (Gasque *et al.* 2000). The importance of neuroinflammation in HD is underscored by human positron-emission tomography studies showing microglial activation in premanifest and early HD patients (Pavese *et al.* 2006; Tai *et al.* 2007).

Clusterin and A2M are intimately involved in complement regulation and both have links to pathogenesis in neurodegeneration. Complement components are synthesized locally in AD brain, and brain-derived clusterin is an inhibitor of MAC formation (Choi-Miura *et al.* 1996; Tai *et al.* 2007). Clusterin and complement component mRNA are upregulated in HD brain and localize to sites of severe pathology including the caudate (Duguid *et al.* 1989; Hodges *et al.* 2006). In AD, clusterin colocalizes with amyloid β (Aβ) in neuritic plaques, is found reversibly complexed with Aβ in CSF and inhibits the aggregation of Aβ *in vitro* (Choi-Miura *et al.* 1996). Clusterin levels are elevated in CSF in AD (Nilselid *et al.* 2006). Clusterin has been shown to cross the blood-brain barrier (Duguid *et al.* 1989) and our data confirm that its concentration in CSF rises in parallel with that in plasma in HD.

A2M is upregulated in reactive astrocytes during brain injury (Du *et al.* 1998). It binds strongly to Aβ, localizes to senile plaques in AD, is neuroprotective to cells exposed to Aβ toxicity and mediates Aβ degradation and clearance by Endocytosis (Narita *et al.* 1997; Du *et al.* 1998). A link with HD pathogenesis is plausible: both huntingtin fragments in HD and β-amyloid aggregates in AD are ubiquitinated (Gutekunst *et al.* 1999), and A2M-bound proteins are lysed intracellularly (Borth 1992). A2M, along with complement proteins, was recently shown by proteomic analysis to be significantly elevated in plasma in Alzheimer's disease (Hye *et al.* 2006).

Among the non-inflammatory proteins identified, alpha-2-antiplasmin is neuroprotective against excitotoxic neuronal death and has been suggested to have therapeutic potential in stroke (Campbell *et al.* 2004). Afamin, which showed decreased production with advancing HD stage, has binding affinity for vitamin E and has also been shown to be neuroprotective against oxidative stress (Heiser *et al.* 2002).

III.5.3 Identification of novel biomarker candidates

The proteins identified by our discovery experiments may have the ability to function individually or together as biomarkers of HD. An ideal HD biomarker would directly measure a disease process occurring in the brain, but there is a valuable role for peripheral biomarkers of neurodegenerative diseases, over and above the ready accessibility of plasma compared with brain tissue or CSF. First, some of the changes seen in plasma may be due to CNS processes causing direct leakage into plasma. The possibility of identifying brain-specific isoforms in plasma to study this warrants further investigation. Second, a single mechanism arising from the HD triplet repeat expansion may produce parallel changes in both central and peripheral tissues, causing plasma levels of a protein to mirror those in the CNS. Third, as discussed above, peripherally produced molecules may exert central effects on the brain.

In order to function as desired, to reduce duration, cost and sample size requirements for clinical trials of potential disease-modifying treatments, a candidate biomarker must be obtainable from easily accessible biofluid, must track linearly with disease progression and should have mechanistic links to disease pathogenesis in order to respond predictably to disease modification (Henley *et al.* 2005). The proteins highlighted by this study appear to meet these basic requirements. Clusterin, in particular, which was evaluated by two techniques in two independent populations in both plasma and CSF, has promise as a biomarker for HD. The mouse IL-6 data also suggest a possible translational biomarker for testing therapeutic candidates across species, though the non-linear pattern of change in both human and mouse likely reflects the multifactorial regulation of neuroinflammatory pathways.

For large scale validation and later clinical use, it is likely that a combination of several clinical, neuroimaging and biochemical biomarkers will be necessary to track disease progression in HD (Henley *et al.* 2005). The potential markers identified here warrant further investigation in large longitudinal cohort studies, as well as in concert with potential disease-modifying interventions.

III.6 Publication relating to this chapter

The work presented in this chapter was published as Dalrymple A / Wild EJ *et al.* (2007) *Proteomic profiling of plasma in Huntington's disease reveals neuroinflammatory activation and biomarker*

candidates. **Journal of Proteome Research** 6(7): 2833-2840. All figures in this chapter are reproduced from this article by permission of the American Chemical Society.

Chapter IV A pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease

IV.1 Introduction

MUTANT HUNTINGTIN IS expressed ubiquitously (Sathasivam *et al.* 1999) and HD causes numerous abnormalities outside the central nervous system (CNS), including upregulation of immune proteins (Björkqvist *et al.* 2006; Robbins *et al.* 2006; Van Raamsdonk *et al.* 2007). The interactions between CNS pathology and changes detectable in peripheral tissues in HD are poorly understood, but may be of importance in measuring or slowing disease progression. The work presented in Chapter IV demonstrates evidence of immune activation in peripheral plasma in manifest HD identified using proteomic profiling but no significant differences between controls and premanifest mutation carriers have previously been shown.

The nature of the immune activation in HD remains incompletely explored. It is not known whether the innate or adaptive arm of the immune system, or both, is activated in HD, and the alterations in each immunomodulatory cytokine at each disease stage are unknown. The cause of the immune activation peripherally is also unknown. IL-6, which triggers the acute phase response, is produced primarily by monocytes and lymphocytes but this could be due to dysfunction of these cells caused by expression of mutant huntingtin (i.e. a cell-autonomous effect) or in response to inflammation-triggering events outside these cells, such as huntingtin-induced tissue damage or the mutant protein itself being interpreted as an antigen (i.e. non-cell-autonomous pathways). Of interest in this respect is the finding that the IκB kinase / NFκB signalling pathway that triggers IL-6 release is upregulated by mutant huntingtin and this may contribute to neurotoxicity (Khoshnan *et al.* 2004).

Critically, the relationship between peripheral inflammation and CNS pathology in HD is unknown. Certain inflammatory proteins, such as complement proteins and clusterin, are also upregulated both peripherally and in the brain in HD (Gasque *et al.* 1995; Hodges *et al.* 2006 and chapter IV). In vivo imaging, in vitro and post-mortem studies have shown that microglia — the CNS counterpart of macrophages — are activated in premanifest (Tai *et al.* 2007) and manifest HD (Sapp *et al.* 2001), that microglial activation correlates with disease severity (Pavese *et al.* 2006) and that mutant huntingtin is expressed in microglia (Shin *et al.* 2005). Thus, inflammation is an established, though

incompletely understood, feature of HD with likely pathogenic importance. Inflammatory changes in the CNS and peripheral tissues in HD may be due to independent effects of mutant huntingtin in both compartments, causing analogous derangements centrally and peripherally; or inflammatory activation may begin peripherally and spread to the CNS — or vice versa — through the passage of immunomodulatory molecules across the blood-brain barrier.

Insights into CNS and peripheral immune system interactions in HD may provide new biomarkers and improve knowledge of key pathogenic mechanisms, possibly leading to novel therapeutic approaches. The present work seeks to elucidate further the nature of the peripheral inflammatory activation in HD, through quantification of levels of key inflammatory and immunomodulatory molecules in human plasma and serum from 3 different mouse models of HD. To investigate possible links between peripheral inflammation and neuronal dysfunction, associations were examined between individual inflammatory molecules and clinical features of HD. Targeted transcription profiling was used to examine expression of key immunomodulatory proteins in the HD striatum, to determine whether the inflammatory activation seen peripherally is mirrored in the CNS. Expression of huntingtin in its wild-type and mutant forms was examined in human monocytes to investigate the possibility that the immune activation is due to a disease-related cell-autonomous dysfunction of these cells. This was confirmed with functional studies of human monocytes, and macrophages and microglia from HD mouse models, demonstrating that there is disease-related dysfunction of CNS and peripheral inflammatory cells in HD.

The chemokine system is a family of some fifty small molecules and twenty receptors, related to but distinct from cytokines and having in common roles as leukocyte chemoattractants. Chemokines are now recognised as central to many processes related to infection and immunity, including migration of leukocytes into the CNS and modulation of the function of the blood-brain barrier. Quantification of chemokine levels may shed light on the status of both the immune system as a whole and the function of specific immune components (Cardona *et al.* 2008).

Chemokine levels were determined in plasma samples using the same multiplex ELISA platform. Associations with clinical scores were examined, and an analysis was performed to examine the possible utility of chemokines as cross-sectional biomarkers, both alone and as a source of additional information in combination with cytokines.

IV.2 Contributions and collaborations

Multiplex ELISA and immunoglobulin quantification was performed by M Björkqvist and A Magnusson. RT-QPCR studies were performed by C Benn and G Bates. Monocyte sorting and stimulation experiments were performed jointly by the author with M Lowdell, R Andre and N Lahiri. R6/2 and knock-in animals were reared by B Woodman and G Bates. YAC128 husbandry and experiments were performed by J Thiele and B Leavitt. Microglial stimulation was performed by E Raibon, R Lee and T Möller. Striatal expression experiments were performed by A Sylvestroni and T Möller. Collection and processing of matched CSF and plasma was performed by B Leavitt. Figure 36 was prepared by D Soulet. Subject recruitment and clinical characterisation, plasma processing, statistical analysis of all data, preparation of all other figures and drafting of the published manuscript were performed by the author. Study conception, design and interpretation was led by the author, M Bjorkqvist and S Tabrizi with contributions from all collaborators. Revision of the manuscript was shared between all collaborators including the author.

IV.3 Subjects and methods

IV.3.1 Ethical approval and subject recruitment

Ethical approval (including animal experiments) and subject recruitment were as specified in Chapter II.

IV.3.2 Inclusion and exclusion criteria

Subjects with concomitant CNS disorders, significant medical comorbidity, known liver dysfunction, recent alcohol or substance abuse, and those taking medications or supplements suspected or known to interfere with the experimental methods used, and subjects with inflammatory or infective conditions were excluded. Patients with advanced HD were not included in this work.

IV.3.3 Collection and processing of human plasma samples

Subjects were classified as controls, premanifest HD or early or moderate manifest HD. Sample collection, fractionation and storage and CAG repeat sizing were carried out as outlined in Chapter II. A subset of subjects was assessed on the unified Huntington's disease rating scale (The Huntington Study Group 1996) by a neurologist experienced in assessment of HD patients. Subjects' demographic and clinical data are given in Table 8.

Experiment	Disease stage	Subject numbers	Female:male	Mean age (range)
Cytokine multiplex assay	Control	69	43:26	42 (22-67)
Plasma	Premanifest	34	19:15	39 (23-54)
	Early	47	24:23	47 (25-80)
	Moderate	44	30:14	52 (38-76)
IgA, IgM assay	Control	26	18:8	46 (29-65)
Plasma	Premanifest	11	5:6	39 (27-49)
	Early	17	9:8	47 (31-61)
	Moderate	18	14:4	50 (26-76)
IgG assay	Control	19	11:8	40 (26-67)
Plasma	Premanifest	17	9:8	38 (27-47)
	Early	13	8:5	42 (25-60)
	Moderate	20	11:9	51 (38-74)
GM-CSF assay	Control	24	20:4	37 (22-67)
Plasma	Premanifest	24	15:9	38 (27-54)
	Early	24	14:10	48 (25-80)
	Moderate	28	17:11	51 (26-77)
IL-6 and IL-8 assay	Control	9	6:3	45 (25-66)
Matched CSF and plasma	Early	9	4:5	51 (38-64)
	Moderate	11	1:10	53 (38-72)
Chemokine plasma assay	Control	34	22:12	44 (25-65)
	Premanifest	15	8:7	39 (23-54)
	Early	23	11:12	47 (31-65)
	Moderate	27	19:8	52 (26-76)

Table 8 Clinical characteristics of subjects in each human biofluid study

Plasma work was performed using a combination of overlapping and unique samples. There was also overlap with the plasma samples used for the work presented in the previous chapter. Paired CSF/plasma samples were from the same subjects as those used for the clusterin ELISA described in the previous chapter.

IV.3.4 Collection of matched CSF and blood samples

30 CSF donors were recruited (Table 8) and their CSF handled as detailed in chapter II.9. These subjects are the same as those reported in Chapter III.

IV.3.5 Collection of mouse serum samples

For the present experiments, *Hdh*$^{Q150/Q150}$ knock-in and R6/2 exon 1 models that develop comparable and widespread molecular phenotypes (Woodman *et al.* 2007) were used. R6/2 (Mangiarini *et al.* 1996) and *Hdh*$^{Q150/Q150}$ mice (original nomenclature: CHL2) (Lin *et al.* 2001) were bred and serum samples collected as previously described (Woodman *et al.* 2007). All animals had unlimited access to water and breeding chow (Special Diet Services) under a 12h light:12h dark cycle. YAC128 mice were maintained on the FVB/N strain background (Slow *et al.* 2003). Numbers and ages of animals are shown in Table 9.

Experiment	Mouse model	Age of animals	Number of WT animals	Number of disease animals
Serum cytokine multiplex assay	R6/2	12 weeks	20	20
	HdhQ150Q/Q150	22 months	10	9
Macrophage stimulation study and serum IL-6 ELISA	YAC128	12 months	3	4
	YAC18	12 months	4	4
Microglial stimulation study	R6/2	Neonatal	4	4

Table 9 Details of animals used for murine experiments

IV.3.6 Serum and plasma analyses

Cytokine levels were quantified using Meso Scale Discovery (MSD) assays as per the manufacturer's protocol and analyzed on a SECTOR™ 2400 instrument (MSD). The operator was unaware of the disease state of each sample during processing and statistical analysis was

performed independently. Serum levels of immunoglobulins (IgG, IgM and IgA) were determined by single radial immunodiffusion assays (The Binding Site Ltd), following the manufacturer's protocol.

IV.3.7 Human monocyte huntingtin expression study

Whole blood was collected from HD patients and controls matched for age and sex (Table 10A). Leukocytes were isolated by density gradient centrifugation over Lymphoprep™ solution (Axis-Shield). Monocytes were obtained by flow cytometry. Briefly, mononuclear cell suspensions were labelled with anti-CD45 FITC and anti-CD14 PE (BD) and viable monocytes sorted flow cytometrically by immunophenotype (CD45+/CD14+) and forward angle light scatter signals (FACSAria high speed cell sorter; BD) to at least 95% purity (see Figure 23A).

A. Huntingtin expression study	Sex	Age	Status	Disease stage (Shoulson et al. 1979)	CAG sizes
C1	F	45.8	Control		
C2	M	43.8	Control		
HD1	M	41.9	HD	2	17/45
HD2	F	44.2	HD	2	25/43
HD3	F	61.4	HD	2	16/42

B. Monocyte functional study	N	Female:male	Mean age (SD)	Mean expanded CAG repeat length (range)
Controls	8	2:7	42.2 (10.3)	
Premanifest HD	9	4:4	38.8 (6.2)	43.5 (41-48)

Table 10 Details of subjects whose blood was used for human monocyte studies

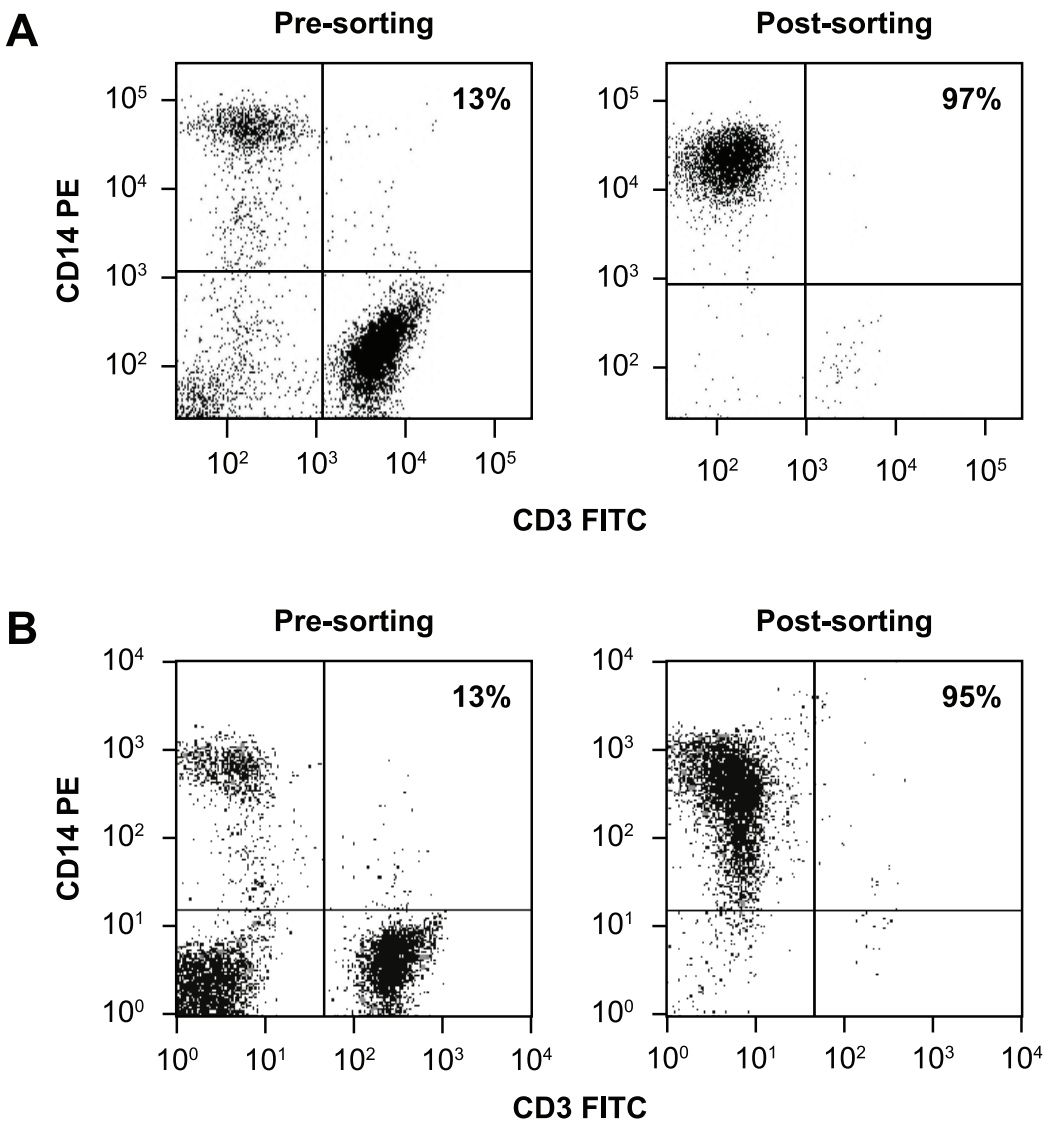


Figure 23 Representative flow cytometry plots

*Plots demonstrate purity of cells obtained by (**A**) flow cytometry and (**B**) magnetic sorting. Monocyte purities are shown as percentages.*

RNA was prepared from pellets of 5×10^6 cells using an RNeasy mini kit (Qiagen) according to manufacturer's instructions. Quality and quantity of RNA was assessed using the RNA nanochip method on a BioAnalyzer (Agilent Technologies). Reverse transcription (RT) of 1µg of total RNA was performed in 50mM KCl, 10mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 6.5mM MgCl2, 10mM DTT, 1mM dNTPs, 10ng/µL random hexamers with 0.35U/µL RNasin (Promega) and MMLV reverse transcriptase (Invitrogen) for 10min at 23°C then 40min at 37°C. The RT reaction was diluted 10-

fold in nuclease-free water (Sigma) and 5µL was used in a 25µL reaction containing Precision Mastermix (PrimerDesign), 300nM primers and 200nM probe. Cycling conditions were: 2min at 50°C, 15 min at 95°C, 44× (1min at 94°C, 1min at 60°C) using the Opticon 2 real-time PCR machine (MJ Research). The threshold used for the analysis was set at 0.05 and reactions were performed in triplicate for each sample. Primer and probe sequences are listed in Table 11. Expression of huntingtin was calculated using $2^{-\Delta\Delta CT}$ with beta-2-microglobulin (B2M) as the reference (Gabert *et al.* 2003). Positive control samples with known B2M expression levels produced consistent results under these experimental conditions.

Gene	Forward primer	Reverse primer	Probe (5′-FAM, 3′-TAMRA)
Abl	TGGAGATAACACTCTA AGCATAACTAAAGGT	GATGTAGTTGCTTGG GACCCA	CCATTTTTGGTTTGGG CTTCACACCATT
Beta 2 microglobulin	GAGTATGCCTGCCGT GTG	AATCCAAATGCGGCA TCT	CCTCCATGATGCTGCT TACATGTCTC
Huntingtin	GCTGCACCGACCGTG AGT	CGCAGGCTGCAGGG TTAC	CAGCTCCCTGTCCCG GCGG
CAG repeat sizing	ATGAAGGCCTTCGAG TCCCTCAAGTCCTTC*	GGCGGCTGAGGAAG CTGAGGA	N/A

Table 11 Primers used for human monocyte expression studies

**FAM-tagged primer.*

CAG repeats were measured in RNA using an ABI3730 automated sequencer and all instruments and materials were obtained from Applied Biosystems unless indicated. The p4G6E4.0 plasmid, which expresses exon 1 of huntingtin with 18 CAG repeats, was used as a positive control. PCR was performed in AM buffer, 10% DMSO, 200µM dNTPs, 10ng/µL primer with 0.5U/µL Taq polymerase (Perkin Elmer). Cycling conditions were 90sec @ 94°C, 25 × (30sec @ 94°C, 30sec @ 68°C, 90sec @ 72°C), 10min @ 72°C. The FAM-tagged PCR product (1µL) together with MegaBACE™ ET900 (Amersham Bioscience) internal size standard (0.04µL) were denatured at 94°C for 5min in 9µL of HiDi formamide. The run conditions were as follows: capillary size, 36cm, Polymer-PoP-7™. The run module was oven temperature, 66°C; buffer temperature, 35°C; pre-run voltage, 15kV; pre-run time, 180sec; injection voltage, 3kV; injection time, 20sec; first readout time, 200msec; second readout time, 200 msec; run voltage, 10kV; voltage number of steps, 10; voltage

step interval, 20sec; voltage tolerance, 0.6kV; current stability, 10μA; ramp delay, 1sec; data delay, 120sec; run time, 2700sec. Data analysis was performed using the plate manager application GeneMapper v5.2-3730XL.

IV.3.8 Functional study of human monocytes

Whole blood was collected in heparin (CP pharmaceuticals) from subjects as detailed in Table 10B. Leukocytes were isolated by density gradient centrifugation over Histopaque-1077 solution (Sigma). Monocytes were obtained by magnetic sorting to increase yield and minimize handling time. Mononuclear cell suspensions were labelled with anti-CD14 microbeads and sorted through magnetic cell separation columns (Miltenyi Biotech) to at least 95% purity (see Figure 23B). Monocytes were counted and 5×10^5 cells per well seeded into 24-well culture plates in RPMI culture medium supplemented with 5% FBS, 2mM L-glutamine and 1% penicillin/streptomycin (Invitrogen). Cells were incubated for 16 hours before stimulation. The medium was then changed to fresh cell culture medium, with or without 10ng/mL IFN-γ (R&D systems). For LPS stimulation, 2μg/ml LPS was added to the medium (Sigma). After 24 hours, supernatants were harvested from two separate wells for each subject/condition. The cells remaining were lysed in 50 mM Tris (pH 8), 150 mmol NaCl, 0.5% sodium deoxycholate and 0.5% Triton X-100, and assayed for total protein concentration using the BioRad protein assay kit following the manufacturer's instructions (BioRad). IL-6 concentrations in supernatants were determined using the MSD assay and adjusted for total protein concentration.

IV.3.9 Functional study of tissue macrophages

Alveolar macrophages were isolated from 12-month-old WT, YAC18 and YAC128 mice, all maintained on a pure FVB/N strain background. The YAC128 (line 53) mouse line expresses high levels of full-length human huntingtin with ~128 polyglutamine repeats and is a well-established model of HD. These mice develop an age-dependent phenotype similar to that seen in HD patients, including cognitive deficits, motor dysfunction, and selective neurodegeneration; 12-month mice are equivalent to early human HD (Slow *et al.* 2003). YAC18 mice (line 212) express transgenic human WT huntingtin and do not exhibit any disease phenotype relative to their WT littermates. They differ from YAC128 mice only in the length of the polyglutamine tract.

Animals were sacrificed using 10mg avertine i/p injection. Blood was drawn from the inferior vena cava and serum samples obtained by two-stage centrifugation. Alveolar macrophages were extracted by intratracheal infusion of ice-cold PBS (Gibco) followed by centrifugation and resuspension of extracted cells. Cells were counted and seeded at 1.5×10^5 cells/mL onto 96-well gelatin-coated plates and incubated in culture media containing 5% medium (RPMI 1640 (Gibco), 5% FBS (Cansera) and 1% penicillin/ streptomycin (Gibco)). After 24 hours this was changed to 1% medium (RPMI 1640, 1% FBS, and 1% P/S). Functional studies were performed after a further 24 hours. The medium was changed to fresh 1% medium, or 1% medium containing 10µg/L IFN-γ (R&D systems) with or without 100µg/L control standard endotoxin (Cape Cod). After 24 hours, IL-6 concentrations were measured in supernatants from two independent wells from each animal for each condition, using a commercial mouse IL-6 ELISA kit following the manufacturer's instructions (eBioscience). Numbers of animals used in each experiment are given in Table 9.

IV.3.10 Functional study of microglia

Mixed primary glial cultures were prepared from single brains of R6/2 mice (B6CBA-Tg(HDexon1)62Gpb/3J; Jackson Labs) as previously described (Weydt *et al.* 2004). In brief, four-day old mice were decapitated; the brains were removed and submerged in ice-cold Hank's saline. The meninges and blood vessels were removed before the tissue was trypsinized, carefully dissociated with a 5ml pipette, resuspended and filtered twice (100µm-diameter Falcon™ filter, BD) before seeding the cells in 5ml of medium per flask (1 brain/flask). Cells were cultured in poly-ornithine-coated $25cm^2$ flasks in DME and supplemented with 10% FBS (D10F).

Littermate heterozygote R6/2 and WT mice were used; each brain was processed and cultured individually to prevent cross-contamination between animals. Genotype and CAG length repeat were determined by PCR from tail samples taken at the time of CNS culture preparation (Laragen). Once astrocytes reached confluence (5-7 days), D10F medium was supplemented with 2ng/mL of GM-CSF. Microglial cells were collected, pooled according to genotype, and seeded in 96-well Primaria™ plates ($2.5x10^4$ cells in 250µL D10F per well; BD). Cultures were >95% pure as assessed by CD11b immunostaining. For each experiment, WT and R6/2 microglial cells were processed in parallel.

24 hours after plating, the cells were serum-starved (MSFM- 0.2ng/ml GM-CSF) for an additional 24 hours. They were then stimulated with IFN-γ (10U) ± LPS (10ng/ml) or carrier control. After 24h of stimulation, supernatant was collected and stored at -80°C for further analysis.

IL-6 concentration was measured by Luminex bead array system (Qiagen). 60µL of supernatant of 3 representative experiments (n=4 for each condition) was thawed and processed using the BioPlex™ platform (BioRad).

IV.3.11 Striatal gene expression study

Total RNA was isolated and purified from striatal samples obtained from The New Zealand Neurological Foundation Human Brain Bank and the New York Brain Bank at Columbia University (6 controls and 17 patients with pathological grades as shown in Table 12) with the RNeasy mini kit (Qiagen). mRNA was transcribed into cDNA with SuperScript III (Invitrogen). RT-QPCR was performed in triplicate with target-specific Roche Universal Library probes (FAM) and Roche universal master mix (Roche Diagnostics), and analyzed with an ABI PRISM® 7500 RT-QPCR System. Mitochondrial ribosomal protein S35 (MRPS35) expression was determined by duplex PCR (VIC) in the same sample to normalize target expression to a housekeeping gene. MRPS35 was chosen based on gene array data showing it not to be regulated in HD striatum (Dr. A. Strand, FHCRC, personal communication). The target/MRPS35 ratio was used to compare the relative target expression using the modified $\Delta\Delta C_T$ method (Pfaffl 2001). Purity of mRNA was checked by performing qPCR without prior RT. Stability of expression of the housekeeping gene MRPS35 was confirmed by comparison with a second house keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Group	n	Female: male	Mean age at death (Range)	Mean CAG repeat length (SD)	Relative RNA quantification, % of controls (SD)		
					IL-6	IL-8	TNF-α
Control	6	0:6	59 (42-74)		100 (88.3)	100 (40.1)	100 (60.8)
HD	17	3:14	54 (40-74)	45 (5)	1520 (159)	1110 (125)	208 (110)
VS1	4	0:4	50 (41-58)	41 (3)	4220 (147)	596 (108)	382 (235)
VS2	4	0:4	62 (47-74)	45 (4)	694 (122)	402 (73.6)	92.1 (153)
VS3	4	0:4	54 (40-64)	45 (4)	3210 (195)	1950 (152)	306 (75.8)
VS4	5	3:2	49 (45-53)	53 (4)*	775 (218)	2740 (36.7)	684 (57.7)

Table 12 Demographic, clinical and pathological characteristics of subjects in the post-mortem striatal expression study

VS, Vonsattel pathological grade (Vonsattel et al. *1985). *CAG repeat lengths available for 2 samples only.*

IV.3.12 Statistical analysis

For the human plasma cytokine and immunoglobulin data, inter-group differences were identified by one-way ANOVA with post-hoc Tukey HSD testing to allow for multiple comparisons. Linear regression analysis using coded variables for each subject group (control=1, premanifest=2, early=3, moderate=4), using age and sex as covariates, was used to identify significant change with advancing disease (Dalrymple *et al.* 2007).

Calculations of estimated time to onset in premanifest subjects were made using the age- and CAG-dependent conditional onset probability formula of Langbehn *et al.* (Langbehn *et al.* 2004).

Correlations with clinical variables were examined using linear regression analysis and partial correlations. Because the distribution of UHDRS and TFC data was not Gaussian, bootstrapping with 1000 replications was used in order to enable linear regression analysis and the use of age as a

covariate. This analysis inevitably involved the use of multiple statistical tests, but because the associations under investigation were of independent scientific interest *p*-values were not corrected for multiple comparisons (see chapter II and Savitz *et al.* 1995).

To examine for the ability of combinations of plasma cytokines to distinguish between different subject groups, stepwise logistic regression was used (Teunissen *et al.* 2003). The model tested the 6 cytokines with statistically significant correlations across disease stage, in order of diminishing R-value (IL-6, IL-8, IL-4, IL-10, TNF-α and IL-5; see Figure 24B); variables were removed from the model when $p<0.05$ for the logistic regression. The comparisons assessed were: controls versus premanifest HD; controls versus all HD expansion-positive subjects; and premanifest versus manifest HD.

ROC curves were constructed using the same method to determine which combinations of chemokines best predicted membership of the same paired groups. For the subjects in whom chemokines were measured, chemokines alone, cytokines alone and the two marker types combined were tested.

Unpaired two-tailed t-tests were used to identify significantly different serum levels for each cytokine in mouse serum and to compare mRNA levels (expressed as $2^{-\Delta\Delta CT}$) between controls and HD patients in the two gene expression studies. Unpaired one-tailed t-tests were used to test the hypotheses that monocytes, macrophages and microglia produce more IL-6 than WT animals when stimulated with LPS.

IV.4 Results

IV.4.1 Cytokine levels

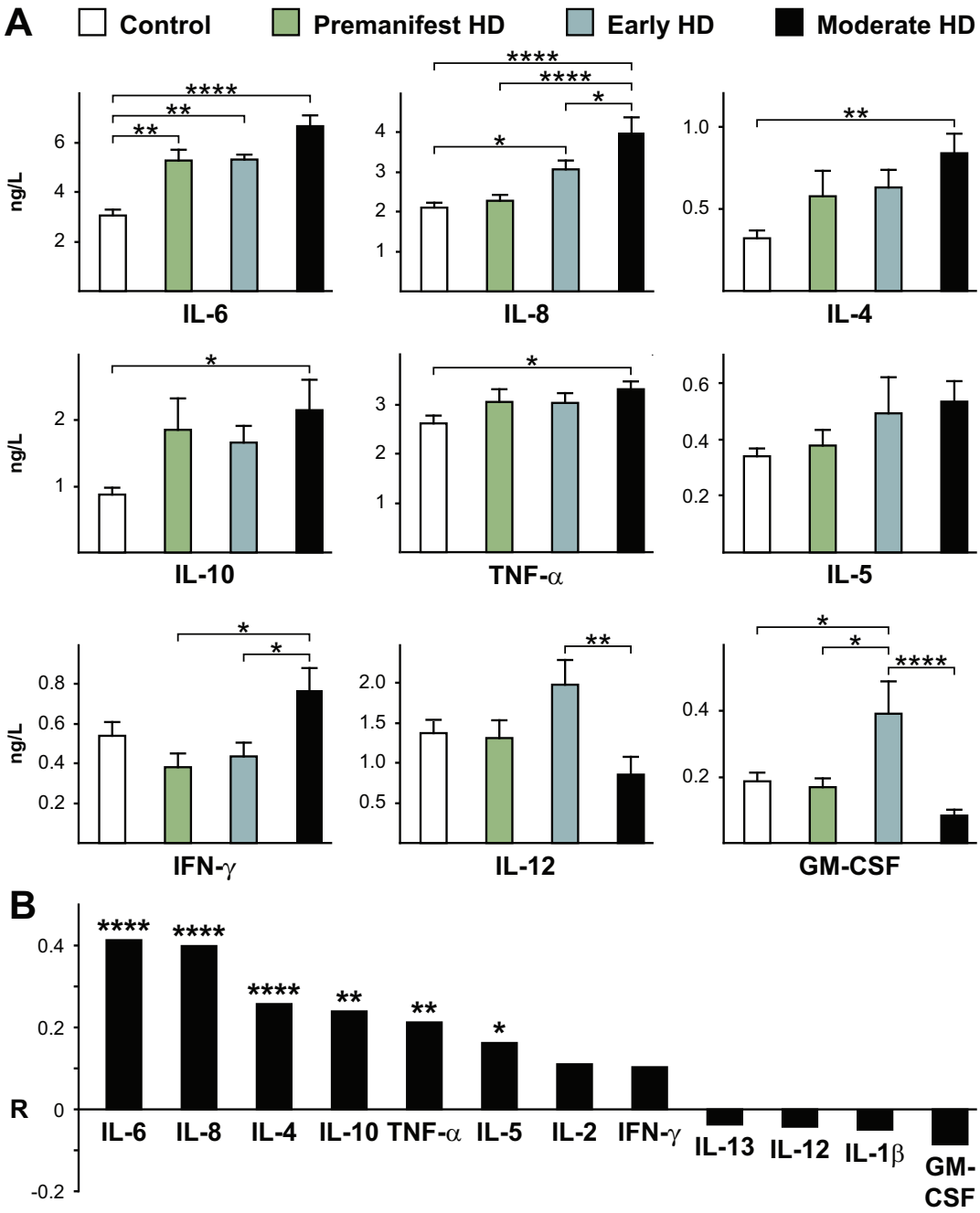


Figure 24 Altered immune profile peripherally in Huntington's disease

A. *Multiplex ELISA quantification of cytokine levels in plasma from HD patients (premanifest, early and moderate HD stages) compared with control subjects. Graphs show mean concentrations with standard error bars. Significant differences between individual groups are shown (ANOVA with post-*

hoc Tukey HSD test). ***B****. The overall trend for increasing levels of cytokines across all groups, analyzed using linear regression, was highly significant for IL-6 and IL-8 and significant for IL-4, IL-10, TNF-α and IL-5. R-values (partial correlation coefficients) are corrected for age and sex.* *p<*0.05,* **p<*0.01,* ***p<*0.001,* ****p<*0.0001. (Raw data supplied by M Björkqvist.)*

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194 plasma samples were collected from HD mutation carriers ranging from premanifest to moderate HD and from control subjects (Table 8) and levels of key inflammatory and immunomodulatory molecules were quantified using multiplex sandwich ELISAs and single radial immunodiffusion assays. An altered profile of cytokine levels was found in HD patients (Table 13). The most striking increases across subject groups, from controls to progressing disease, were in IL-6 and IL-8 (p<0.0001 in each case). In addition IL-4, IL-10 and TNF-α levels increased significantly with disease progression (Figure 24B). Moreover, IL-6 levels were significantly increased in premanifest subjects, who had an estimated mean of 16 years until motor onset (Figure 24A) (Langbehn *et al.* 2004). Interestingly, the cytokines that were increased earliest in the disease course (IL-6 and IL-8) are involved in the innate immune response (Kindt *et al.* 2006). IL-10 and IL-4, anti-inflammatory cytokines involved in the adaptive immune response, increased significantly in moderate stage disease.

	Control		Premanifest HD		Early HD		Moderate HD	
	Mean (SD)	n	Mean (SD)	N	Mean (SD)	n	Mean (SD)	n
IL-6	3.06 (2)	69	5.26 (2.66)	34	5.3 (3.01)	47	6.65 (3.46)	46
IL-8	2.11 (1.03)	69	2.27 (0.97)	34	3.08 (1.47)	47	3.98 (2.67)	46
IL-4	0.32 (0.38)	68	0.58 (0.88)	34	0.63 (0.71)	45	0.84 (0.78)	45
IL-10	0.87 (0.87)	66	1.85 (2.75)	34	1.67 (1.68)	45	2.15 (3.06)	44
TNF-α	2.62 (1.25)	69	3.04 (1.51)	34	3.03 (1.27)	47	3.32 (1.05)	46
IL-5	0.34 (0.23)	66	0.38 (0.33)	34	0.49 (0.88)	45	0.53 (0.5)	46
IL-2	0.86 (0.99)	66	1.06 (0.79)	34	1.01 (0.96)	45	1.51 (4.63)	46
IFN-γ	0.54 (0.58)	66	0.38 (0.39)	34	0.44 (0.44)	45	0.77 (0.76)	46
IL-13	3.17 (2.62)	66	1.84 (1.46)	34	2.48 (2.23)	45	2.35 (12.49)	46
IL-12	1.38 (1.11)	66	1.31 (1.32)	34	1.97 (2.11)	45	0.85 (1.5)	45
IL-1β	0.83 (0.82)	69	0.81 (0.87)	34	0.85 (0.89)	47	0.74 (0.77)	46
GM-CSF	0.19 (0.16)	23	0.17 (0.12)	24	0.39 (0.48)	24	0.08 (0.1)	36
IL-6	3.06 (2)	69	5.26 (2.66)	34	5.3 (3.01)	47	6.65 (3.46)	46
IL-8	2.11 (1.03)	69	2.27 (0.97)	34	3.08 (1.47)	47	3.98 (2.67)	46
IL-4	0.32 (0.38)	68	0.58 (0.88)	34	0.63 (0.71)	45	0.84 (0.78)	45
IL-10	0.87 (0.87)	66	1.85 (2.75)	34	1.67 (1.68)	45	2.15 (3.06)	44

Table 13 Plasma cytokine levels in HD, by disease stage, measured by multiplex ELISA assay

All cytokine levels are in ng/L. (Raw data supplied by M Björkqvist.)

IV.4.2 Immunoglobulin levels

There was no difference in levels of immunoglobulins (IgG, IgA or IgM) at any disease stage (Figure 25), arguing against widespread activation of the adaptive humoral immune system.

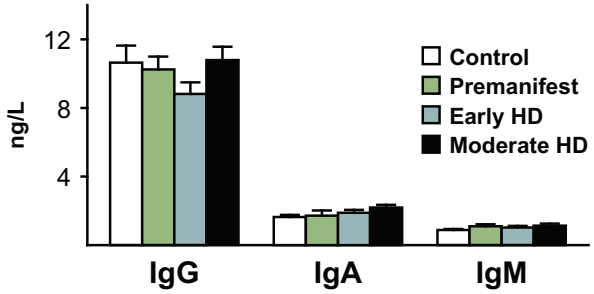

Figure 25 Plasma immunoglobulin levels are unchanged in HD

Quantification of plasma IgG, IgA and IgM by single radial immunodiffusion assays revealed no difference in immunoglobulin levels across disease stages, arguing against widespread activation of the adaptive immune system. Graphs show mean concentrations and standard error bars. (Raw data supplied by M Björkqvist.)

IV.4.3 Associations between cytokines and clinical characteristics

Associations were then examined between these cytokine changes and clinical characteristics. Plasma IL-8 levels increased markedly with disease progression (Figure 24) and correlated with clinical measures of HD. Levels of IL-8 correlated positively with worsening disease, as demonstrated by UHDRS chorea scores (R=-0.37, $p<0.01$) and total motor scores (R=0.27, $p<0.05$), as well as negatively with total functional capacity (TFC) scores where lower scores indicate more severe disease (R=-0.28, $p<0.05$) (Figure 26A). TNF-α levels in plasma correlated with UHDRS chorea scores (R=0.28, $p<0.01$) and UHDRS motor scores (R=0.26, $p<0.05$) (Figure 26B); a negative correlation with TFC was seen that approached statistical significance (R=-0.20, $p=0.07$).

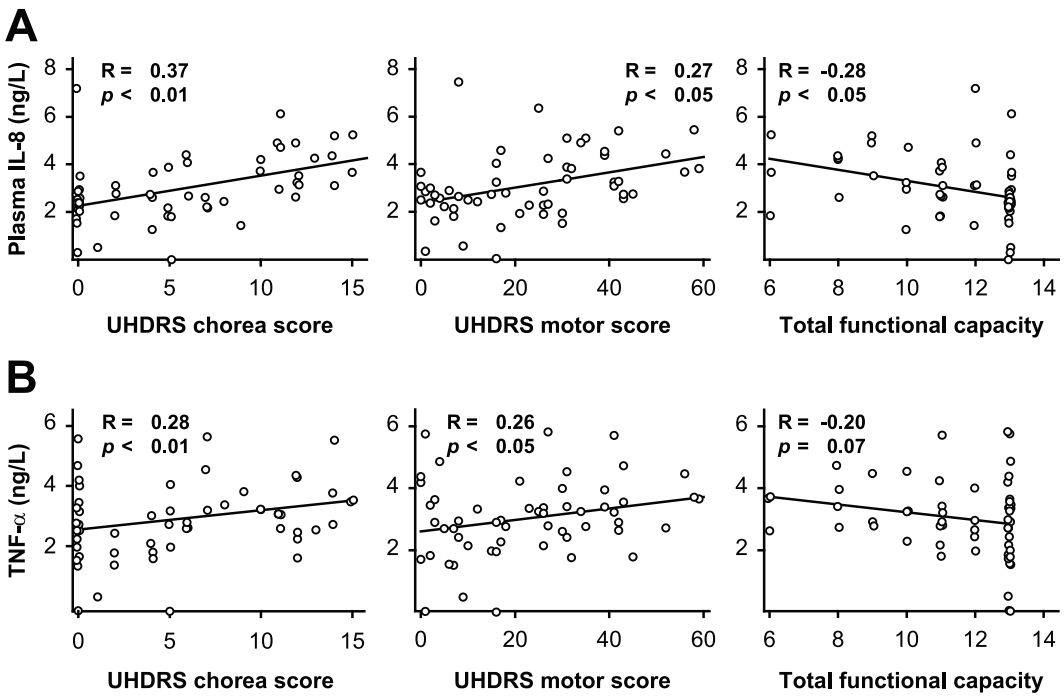

Figure 26 Correlations between plasma cytokine levels and clinical severity scores in premanifest and manifest HD gene carriers

*Levels of (**A**) IL-8 and (**B**) TNF-α correlated with worsening disease as demonstrated by increasing UHDRS chorea and total motor score, and decreasing total functional capacity score. (Raw cytokine data supplied by M Björkqvist.)*

IV.4.4 Cytokines as potential biomarkers

Objective markers of HD progression are needed to facilitate the conduct of clinical trials of disease-modifying therapies in HD. Stepwise logistic regression analysis was used to construct receiver operating characteristic (ROC) curves that demonstrated a strong ability of combinations of plasma cytokine levels to discriminate between disease groups (Figure 27). A combination of IL-6, IL-10 and IL-5 was found to discriminate optimally between premanifest HD and controls with an area under the curve (AUC) of 0.81. A combination of IL-6, IL-8 and IL-10 best discriminated all HD expansion carriers (premanifest and manifest) from controls (AUC 0.82). IL-6, IL-8 and IL-10 together best discriminated between premanifest and manifest HD patients (AUC 0.85). A detailed explanation of ROC curves is given in chapter II.11.11.

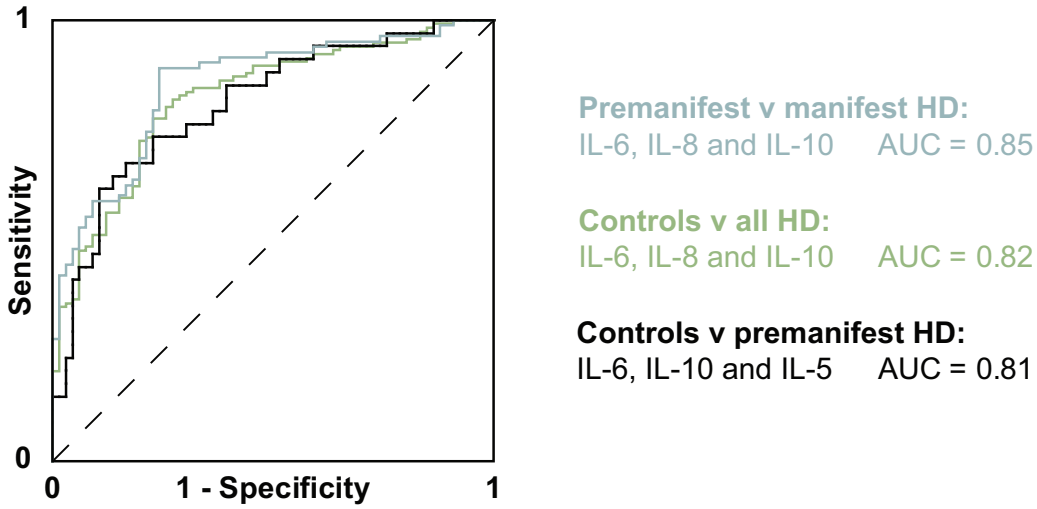

Figure 27 ROC curves demonstrating the ability of different combinations of plasma cytokine levels to discriminate between subject groups

In a ROC curve plot, the 'true positive' diagnosis rate (sensitivity) is plotted against the 'false positive' diagnosis rate (1-specificity) for a test with a binary outcome. The area under the curve (AUC) summarizes the discrimination of the test, i.e. its ability to classify cases correctly. A perfect test would have an AUC of 1, a worthless test an AUC of 0.5. AUC values may be classified as follows: 0.9-1='excellent'; 0.8-9='good'; 0.7-0.8='fair', 0.6-0.7='poor'; 0.5-0.6='fail' (Hanley et al. *1982). For the present analysis, optimum combinations were identified by stepwise logistic regression analysis using a threshold of* p=*0.05 for each cytokine removed from the model. A combination of IL-6, IL-10 and IL-5 best discriminated between controls and premanifest HD; a combination of IL-6, IL-8 and IL-10 best*

discriminated manifest from premanifest HD; and a combination of IL-6, IL-8 and IL-10 best discriminated controls from HD gene carriers (both premanifest and manifest). . (Raw cytokine data supplied by M Björkqvist.)

IV.4.5 Huntingtin expression by human monocytes

IL-6 triggers the acute phase response and is produced primarily by monocytes/macrophages and lymphocytes (Kindt *et al.* 2006). In order to define the possible source of peripheral cytokines, monocytes were purified from whole blood from HD patients and control subjects by flow cytometric and magnetic sorting. Using RT-QPCR, monocytes from HD patients were found to express mutant huntingtin (Figure 28).

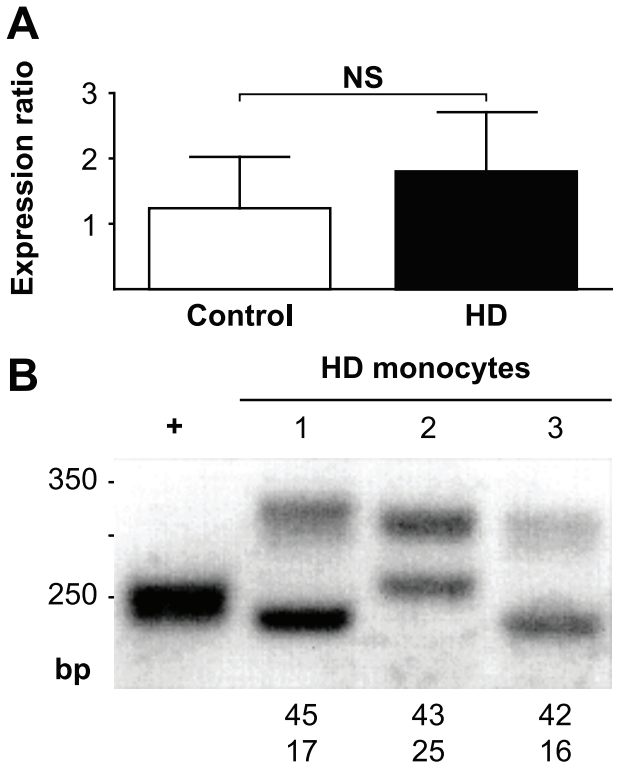

Figure 28 Human monocytes express wild-type and mutant huntingtin

A. *RT-QPCR studies of human monocytes obtained by flow cytometry demonstrated huntingtin expression in 100% of monocyte samples tested from controls (n=2) and HD patients (n=3). Expression ratios are relative to beta-2-microglobulin. Graph shows mean expression ratios with standard error bars.* ***B***. *PCR amplification of CAG repeat tracts from huntingtin mRNA reveals that HD monocytes express both WT and mutant huntingtin, supporting the possibility of cell-autonomous*

dysfunction resulting in immune activation. WT and mutant CAG repeat lengths are shown. +, p4G6E4.0 plasmid, expressing HTT *exon 1 with 18 CAG repeats. . (qPCR data supplied by C Benn.)*

IV.4.6 Functional study of myeloid cells in HD

A series of stimulation experiments was performed to examine the function of myeloid cells in HD. Isolated monocytes from premanifest HD mutation carriers were stimulated with LPS and found to behave abnormally, displaying excess IL-6 production compared with cells from control subjects (Figure 29A). Mutant huntingtin thus appears to produce functional overactivity of monocytes.

To determine whether the presence of the mutant protein causes this dysfunction, isolated macrophages from the YAC128 mouse model of HD were stimulated with LPS. Echoing the results seen in human HD monocytes, macrophages from the YAC128 responded with enhanced secretion of IL-6 in response to stimulation, compared with macrophages from wild-type mice (Figure 29B). YAC128 cells differ from WT cells in respects other than the presence of the mutant protein, so to test whether the presence of mutant huntingtin *per* se is sufficient to produce dysfunction, this stimulation experiment was repeated in macrophages from the YAC18 mouse, which differs from the YAC128 only in the length of the polyglutamine stretch: excessive IL-6 release was not seen in YAC18 cells (Figure 29C).

To determine whether microglia — the CNS equivalent of monocytes/macrophages — are also dysfunctional in HD, LPS stimulation was performed in microglia isolated from the widely-used R6/2 transgenic mouse model of HD (Mangiarini *et al.* 1996). HD microglia, too, demonstrated hyperactivity in response to stimulation (Figure 29D).

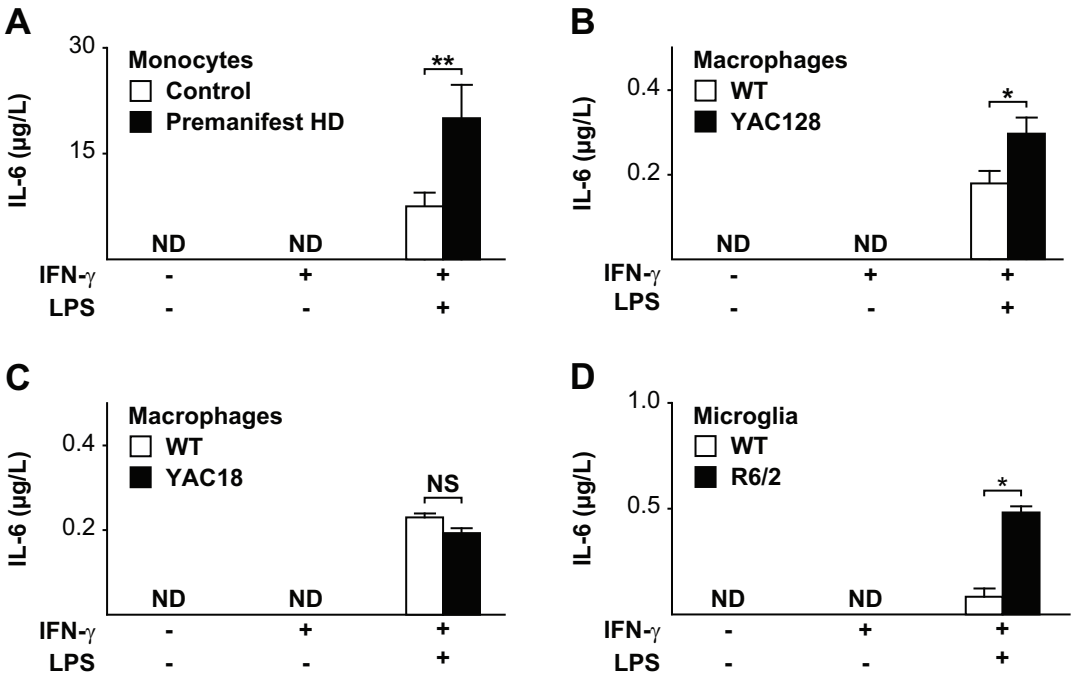

Figure 29 HD monocytes, macrophages and microglia are overactive when stimulated

***A**. No IL-6 was detectable in the supernatant of monocytes from control (n=9) or premanifest HD subjects (n=8) in the unstimulated state or after priming with IFN-γ. Monocytes stimulated by addition of both IFN-γ and 2µg/mL LPS expressed IL-6, but expression levels were significantly higher from HD monocytes. **B**. Alveolar macrophages from the YAC128 HD mouse model have similarly altered function when stimulated. YAC128 macrophages stimulated by addition of both IFN-γ and 100ng/mL LPS expressed significantly more IL-6. n=3 WT and 4 YAC128. **C**. Macrophages from YAC18 mice, which differ from YAC128 cells only in the number of CAG repeats, behaved no differently from WT cells (p=0.231, n=4 per genotype) in response to stimulation at the same LPS concentration, suggesting that the hyperactivity is due to mutant huntingtin. **D**. Microglia isolated from neonatal R6/2 mice are also hyperactive when stimulated by 10ng/mL LPS (n=4 per group). Graphs show mean concentrations with standard error bars. ND, not detected. Unpaired t-tests: *p<0.05; *p<0.01. (Human data produced by the author, M Lowdell, R Andre and N Lahiri; YAC data supplied by J Thiele and B Leavitt; R6/2 data supplied by E Raibon, R Lee and T Möller.)*

IV.4.7 CNS expression of cytokines in HD

Expression of inflammatory transcripts in post-mortem human striatal tissue was investigated using RT-PCR. Markedly increased expression of IL-6, IL-8 and TNF-α was found in HD striatum (Figure 30), mirroring the key changes seen in plasma much earlier in the disease and suggesting *in situ* excess CNS production of these cytokines. Further work, currently under review for publication, has suggested increased striatal expression of IL-10 and, in contrast to the findings from plasma, IL-1β (Dr Thomas Möller, personal communication). Thus while the post-mortem striatum prominently echoes the changes seen in plasma from subjects earlier in the disease, it may not recapitulate them precisely.

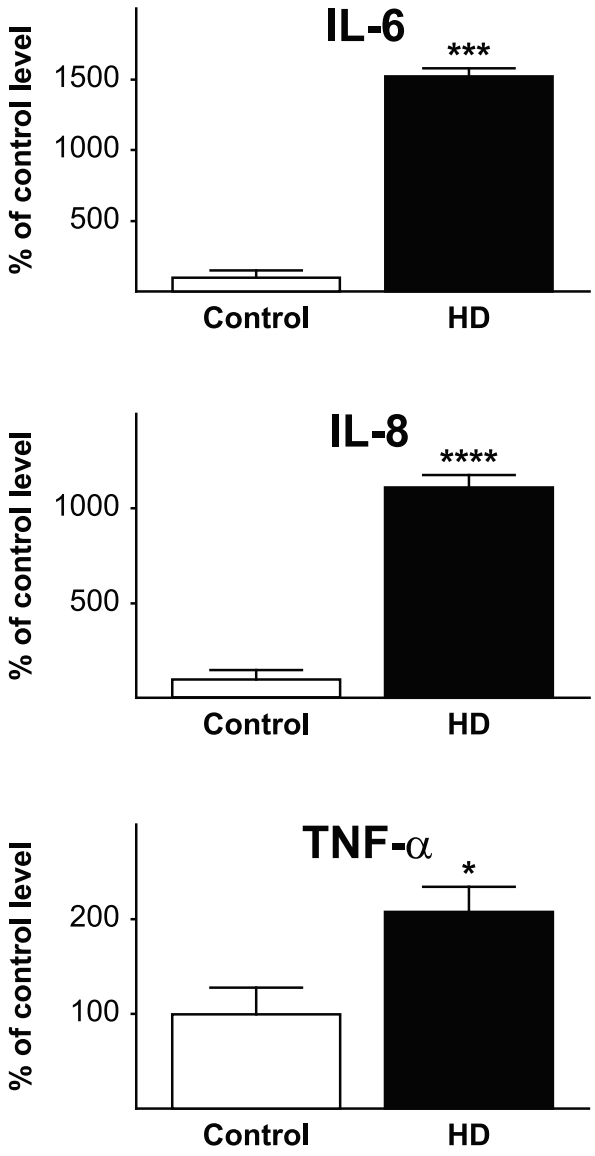

Figure 30 Altered expression of inflammatory transcripts in post-mortem HD striatal tissue

*Levels of IL-6, IL-8 and TNF-α RNA were significantly higher in striatum of HD patients than in control striatum. Graphs show means with standard error bars. n=6 controls and 17 HD patients (see Table 12). Unpaired t-tests: *p<0.05, ***p<0.001, ****p<0.0001. (Raw data supplied by A Silvestroni and T Möller.)*

IV.4.8 Cytokine levels in matched CSF and plasma

To investigate the relationship between central and peripheral inflammatory processes, IL-6 and IL-8 were measured in matched plasma and CSF samples from HD patients and controls using ELISA.

CSF and plasma levels of IL-6 and IL-8 correlated closely (Figure 31; R=0.74 and R=0.66 respectively; p<0.0001 for both).

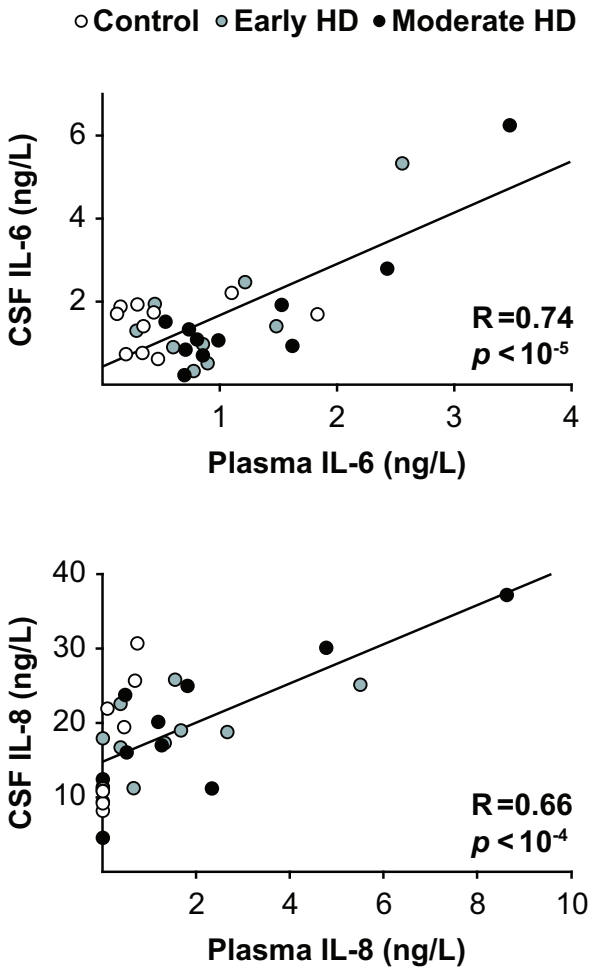

Figure 31 Correlations between matched CSF and plasma levels of IL-6 and IL-8

ELISA-quantified levels in CSF and matched plasma samples correlated strongly for both IL-6 and IL-8. (Raw data supplied by M Björkqvist from samples provided by B Leavitt.)

IV.4.9 Cytokine expression in HD mouse models

Multiplex ELISA was used to determine whether peripheral immune activation is present in serum in HD mouse models. Increased levels of several cytokines were found in the R6/2 transgenic mouse and the full-length *Hdh*$^{Q150/Q150}$ knock-in model of HD (Woodman *et al.* 2007). In 12-week R6/2 mice, IL-1β, IL-6, IL-10 and IL-12p70 were significantly increased (Figure 32A). In 22-month knock-in *Hdh*$^{150Q/150Q}$ mice IL-6, IL-10 and IL-12p70 were significantly elevated (Figure 32B). In the YAC128

mouse model of HD at 12 months of age, similar elevations in serum IL-6 and mKC — a murine functional homolog of IL-8 (Bozic *et al.* 1995) — were seen (Figure 32C). 12-month YAC128 animals are phenotypically equivalent to early HD (Slow *et al.* 2003), and these animals therefore model the human patients whose plasma was studied.

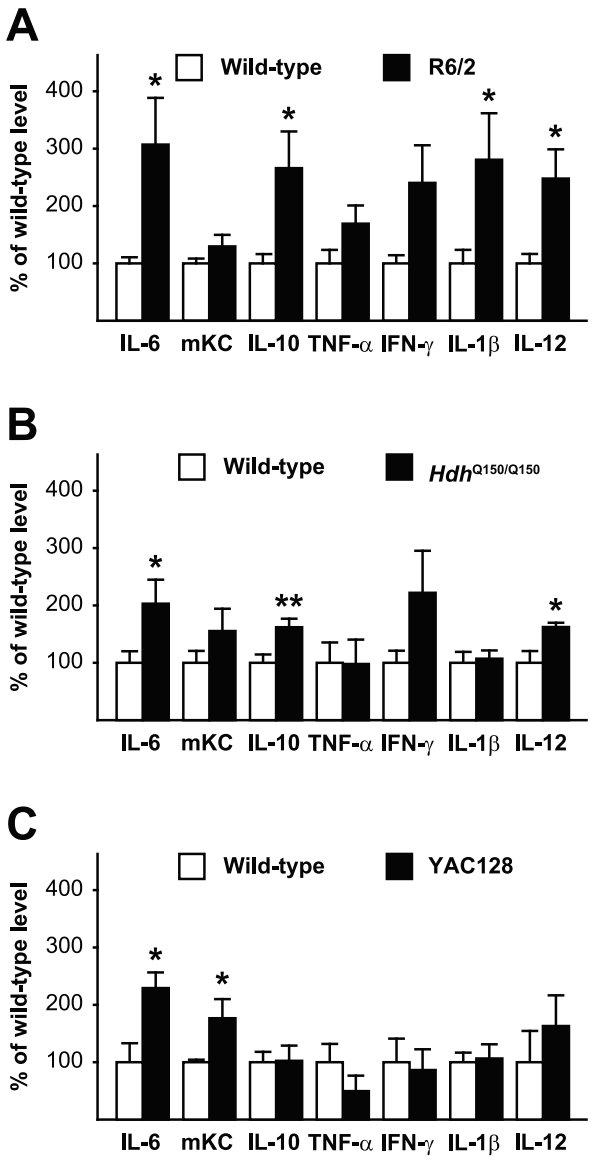

Figure 32 Mouse models of HD recapitulate features of human immune dysfunction

*Serum levels of cytokines, measured by multiplex ELISA, are elevated in both (**A**) R6/2 and (**B**)* Hdh$^{Q150Q/Q150}$ *knock-in mouse models (n=9 per genotype) at end-stage. **C**. In 12-month YAC128 animals (equivalent to early human disease), serum IL-6 and mKC — a murine functional homolog of IL-8 — are significantly increased (n=3 WT and 4 YAC128). Graphs show mean levels with standard*

error bars. Unpaired t-tests: *p<*0.05; ***p<0.01. (Raw data supplied by M Björkqvist from samples provided by B Leavitt, B Woodman and G Bates.)*

IV.4.10 Study of chemokine levels

Chemokine levels were tested in 99 subjects whose demographic characteristics are given in Table 8. As shown in Figure 33, there were significant inter-group differences for all five chemokines tested. For three (eotaxin-3, eotaxin and MCP-4) there were significant differences between adjacent clinical groups. Three chemokines (eotaxin-3, MIP-1β and eotaxin) showed statistically significant increases across all subject groups with advancing disease. When associations between clinical scores and chemokine levels were examined, eotaxin-3 levels were found to correlate with UHDRS motor score ($R=0.41$, $p<0.05$). There were no statistically significant correlations between any other chemokine and UHDRS motor or chorea score or TFC (data not shown).

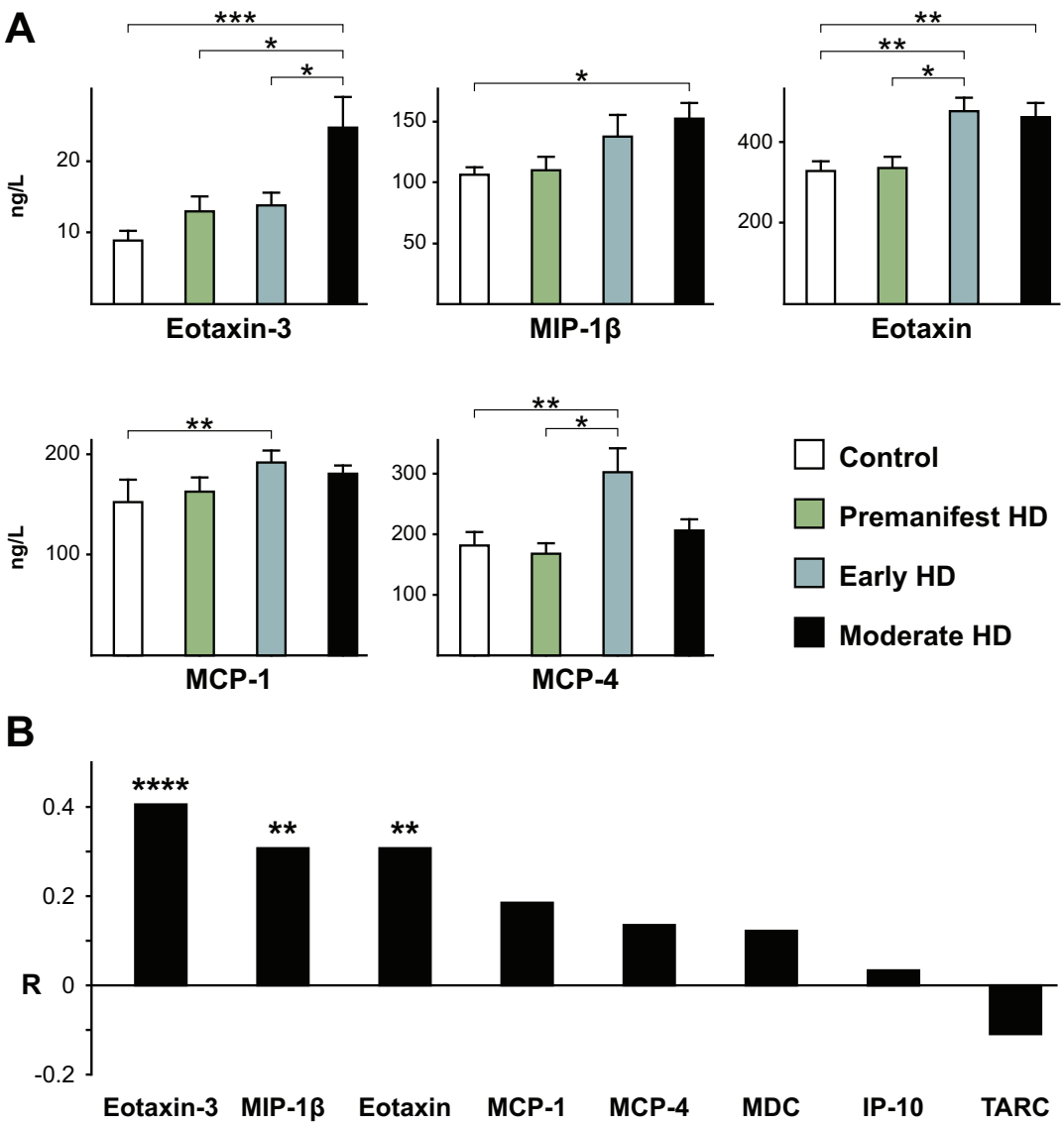

Figure 33 Altered chemokine profile peripherally in Huntington's disease

*(**A**) Multiplex ELISA quantification of chemokine levels in plasma from HD patients and control subjects. Graphs show mean concentrations with standard error bars. Significant differences between individual groups are shown (ANOVA with post-hoc Tukey HSD test). Significant differences between adjacent clinical groups were seen with eotaxin-3 (early vs moderate HD), eotaxin and MCP-4 (premanifest vs early HD). No chemokine levels differed significantly between controls and premanifest HD. (**B**) The overall trend for increasing levels of chemokines across all groups, analyzed using linear regression, was significant for eotaxin-3, MIP-1β and eotaxin. R-values (partial correlation coefficients) are corrected for age and sex. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (Raw data supplied by M Björkqvist.)*

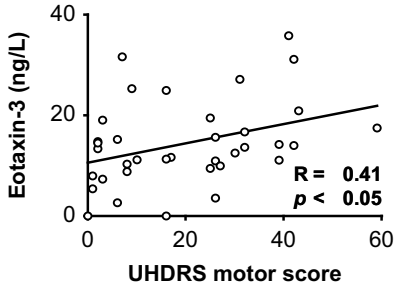

Figure 34 Plasma levels of eotaxin-3 correlate with UHDRS motor score in premanifest and manifest gene carriers

R, partial correlation coefficient corrected for age. (Raw chemokine data supplied by M Björkqvist.)

Stepwise logistic regression analysis was then used to determine which combinations of plasma chemokine levels best predicted membership of different dichotomous subject groupings. The results of this analysis are shown in Figure 35. All five chemokines were used in the model. Each contrast was then repeated using the six 'top' cytokines identified in the previous work, for those subjects in whom both cytokine and chemokine levels were available. Finally, the model was applied using both chemokine and cytokine values, to determine the optimum combination of inflammatory markers for each contrast. For each of the three contrasts tested, the AUC value (a measure of the reliability of the test) was approximately the same as, or lower than, that obtained using cytokines. However, in each case, combining chemokines and cytokines resulted in an optimum combination of markers containing one or more molecules from each category, and an AUC higher than chemokines or cytokines alone.

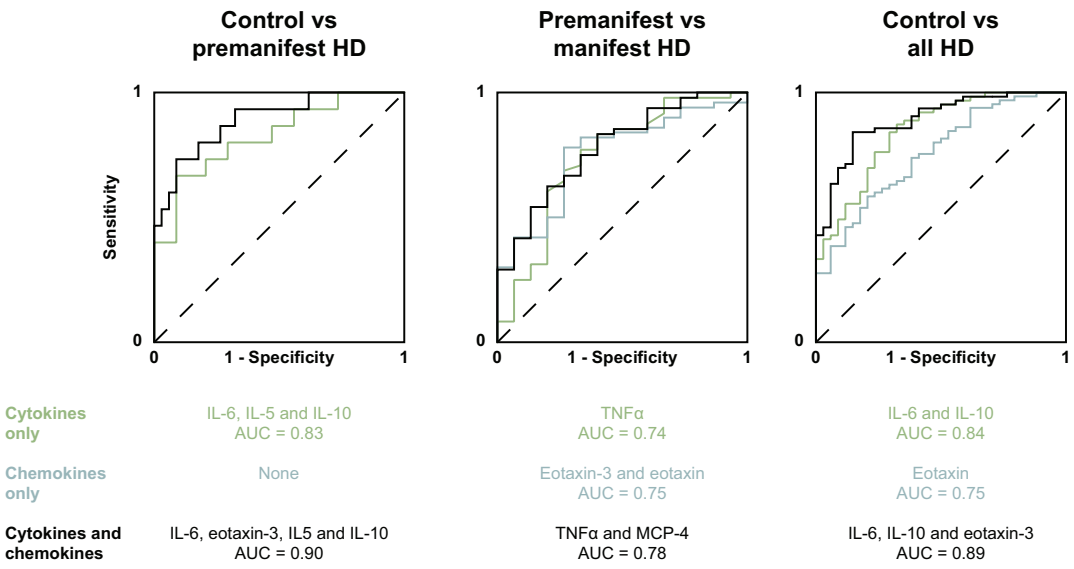

Figure 35 ROC curves demonstrating that chemokine and cytokine levels can be combined to improve the ability of inflammatory markers to distinguish between different clinical groups

The ability of different markers to categorise subjects in each of three pairwise clinical contrasts was tested. For each contrast, ROC curves were constructed using stepwise logistic regression to examine the discriminatory ability of cytokines alone, chemokines alone and both marker types combined. For each set of markers, in each contrast, the markers included in the model and the AUC are given. The threshold for removal from the model was p=*0.05. In every contrast, combining chemokines and cytokines led to an AUC higher than that resulting from either set of markers tested individually. Cytokines tested: eotaxin-3, MIP-1β, eotaxin, MCP-1 and MCP-4. Chemokines tested: IL-6, IL-8, IL-4, TNF-α and IL-5, as above. (Raw chemokine and cytokine data supplied by M Björkqvist.)*

IV.5 Discussion

Taken together, these data show that immune activation in HD is widespread and detectable in peripheral plasma across disease stages. Key cytokines of the innate immune system are upregulated both centrally and peripherally and robust changes even take place in premanifest HD mutation carriers many years before the onset of motor abnormalities. The elevated IL-6 level seen in premanifest subjects with a mean of sixteen years until predicted clinical onset represents the earliest plasma abnormality identified to date in HD. The peripheral changes correlate well with clinical variables and are accompanied by alterations in striatal gene expression.

Although cytokines such as IL-4 and IL-10 are increased later in the disease, normal immunoglobulin levels throughout the disease course suggest that there is no generalized activation of the adaptive immune response. While IL-6 and IL-8 production are triggered by NFκB activation (Fietta *et al.* 2002; Khoshnan *et al.* 2004), IL-4 and IL-10 act to downregulate NFκB (Kindt *et al.* 2006). The late involvement of these cytokines may reflect an adaptive response to chronic immune activation, possibly involving altered interactions between monocytes/macrophages and TH_2 cells.

The correlation shown between plasma and CSF levels of IL-6 and IL-8 links the central and peripheral immune activation in HD. IL-6 and IL-8 are seen to be increased in plasma and the striatum. These cytokines are not thought to cross the healthy blood-brain barrier (BBB) in the acute setting (Steensberg *et al.* 2006; Billiau *et al.* 2007) and studies of brain penetration of specific molecules in HD have not identified alterations of the BBB (e.g. Hersch *et al.* 2006). Mutant huntingtin therefore probably induces parallel dysfunction in both compartments (Figure 36). The immune dysfunction demonstrated here in monocytes from premanifest HD gene carriers may reflect similar central changes in HD microglia, and therefore act as a window onto central disease pathogenesis at this very early stage in the disease process. Given the dramatic changes seen in striatal cytokine expression and the chronic nature of these changes, passage of cytokines from the CNS into blood is a possibility that cannot be excluded, though cytokines are rapidly broken down (Pan *et al.* 2007) and would likely be subject to considerable dilution in plasma. However, the presence of primary abnormalities of both peripheral and central cytokine-producing cells (monocytes/macrophages and microglia respectively) suggests that parallel disease-related derangements in the periphery and CNS are the simplest explanation for these CSF-plasma correlations.

Microglia have previously been implicated in the pathogenesis of HD (Sapp *et al.* 2001; Pavese *et al.* 2006; Tai *et al.* 2007) and are increasingly seen as key players in the pathogenesis of neurodegenerative diseases (Lobsiger *et al.* 2007). The increased cytokine production observed in the CNS and peripherally could be caused by dysfunction of microglia and their counterparts, monocytes and macrophages. These data show that monocytes from HD patients express mutant huntingtin. Further, they demonstrate that microglia, monocytes and macrophages in HD are all hyperactive when stimulated. The NFκB signalling pathway that triggers IL-6 release is known to be

upregulated by mutant huntingtin (Khoshnan *et al.* 2004) and the microglial kynurenine monooxygenase pathway has been identified as a potential therapeutic target in HD, establishing immune dysfunction as a possible pathogenic pathway in HD (Giorgini *et al.* 2005).

The functional overactivity of macrophages from 12-month YAC128 mice — corresponding to early human HD — was not seen in macrophages from YAC18 mice, which are identical to YAC128 animals except for the length of the polyglutamine tract. This suggests that the presence of mutant huntingtin alone is sufficient to cause derangement of monocytes/macrophages.

Interestingly, while IL-6 is undetectable in the supernatant of isolated human monocytes, levels of IL-6 in plasma are not zero in control subjects and HD patients. The same is true of murine macrophages and serum. Thus, there is low-level background cytokine production *in vivo*, perhaps due to normal cell turnover or environmental immune challenges, that is absent *in vitro*. A primary dysfunction of monocytes is sufficient to explain the increased plasma cytokine levels in HD, as hyperactive HD monocytes will respond excessively to this physiological background stimulation. However, it is possible that HD also causes differences in the level of background immune stimulation, which could contribute to the later upregulation of the adaptive immune response cytokines IL-4 and IL-10.

There is a need for markers of progression ('state biomarkers') in HD and other neurodegenerative diseases (Ray *et al.* 2007). These results suggest that inflammatory changes detected in peripheral plasma may be biologically relevant and mirror the neurodegenerative process occurring in the CNS (Figure 36). Indeed, combined peripheral markers of inflammation were recently suggested to be biomarkers for diagnosis and progression in Alzheimer's disease (Ray *et al.* 2007). Remarkably, peripheral inflammatory changes may also reveal early pathogenic events in HD, occurring over 15 years before the onset of neurological manifestations. The study of chemokine levels in HD reveals that levels of inflammatory molecules other than cytokines appear to track with progression and may be able to function as biomarkers, particularly when combined with cytokines, where they appear able to convey additional information and increase the discriminatory power of biomarker combinations.

The inflammatory changes seen in patients are echoed in mouse models of HD. Importantly, they may therefore provide translational biomarkers for the use of HD mouse models in the development of therapeutic interventions. Finally, the mechanism of early innate immune activation in HD warrants further study as a potential source of targets for disease-modifying therapies.

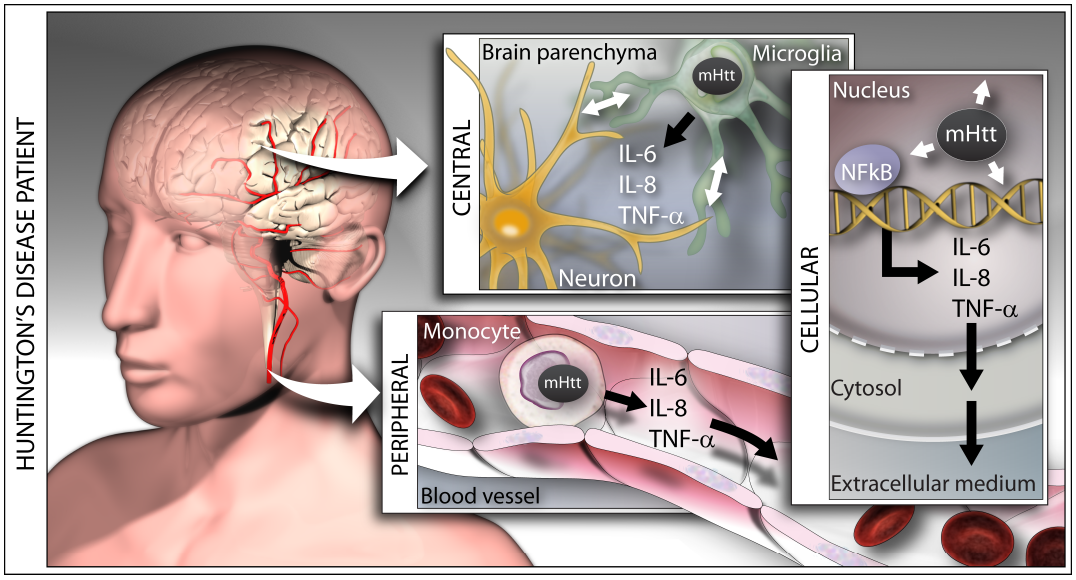

Figure 36 Immune activation, induced by mutant huntingtin, occurs both peripherally and centrally in Huntington's disease

A cell-autonomous effect of the mutant protein may be responsible for the innate immune response. The NFκB signalling pathway that triggers IL-6 release is known to be upregulated by mutant huntingtin (Khoshnan et al. *2004) and microglia-derived toxicity can influence disease progression (Khoshnan* et al. *2004; Giorgini* et al. *2005). This work shows that the innate immune response detectable in plasma very early in the disease is strongly linked to disease progression and recapitulated in HD striatum, that human monocytes express mutant huntingtin and that monocytes, macrophages and microglia overexpress IL-6 when stimulated. Early innate immune activation could be a target in the development of disease-modifying therapies. (Figure prepared by D Soulet.)*

IV.6 Publication relating to this chapter

The work presented in this chapter was published as Björkqvist M / Wild EJ *et al.* (2008) *A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease.*

Journal of Experimental Medicine 205(8): 1869-1877. This publication does not include the chemokine analysis, which is in preparation for submission.

Chapter V Plasma neurofilament levels in Huntington's disease

V.1 Introduction

IN CONTRAST TO the previous two chapters, this chapter focuses on the experimental testing of a specific hypothesis, derived from plasma biomarker work in other neurological diseases, relating to a single candidate biomarker. The need for objective measures that are easy to quantify reliably in accessible tissue or fluid, track linearly with disease progression and change in response to disease-modifying therapeutic interventions has already been discussed and plasma is particularly appealing as a source of biomarkers because it is readily accessible compared with cerebrospinal fluid.

Neurofilament proteins are the dominant proteins of the axonal cytoskeleton. Three neurofilament isoforms (light, L; medium, M and heavy, H) form the neurofilament triple protein (Lee *et al.* 1996). Neurofilament proteins have been established as markers for axonal injury, degeneration and loss and their clinical use as biomarkers has been suggested in a range of acute and chronic neurological diseases including multiple sclerosis, Parkinson's disease and Alzheimer's disease (Petzold 2005). The early course of HD is characterised by neuronal dysfunction rather than neuronal loss (Leegwater-Kim *et al.* 2004). Neurofilament is released by dysfunctioning as well as dying neurons (Petzold 2005), and neuronal loss does occur by the symptomatic stage of HD (Leegwater-Kim *et al.* 2004). Plasma is a desirable target for biomarker identification because of its ready accessibility compared with cerebrospinal fluid. Changes in neurofilament levels have been shown in plasma in other neurological diseases (Petzold 2005). It is therefore reasonable to hypothesise that neurofilament levels may be altered in plasma in HD.

The aim of the present study was to determine whether neurofilament H (NfH) levels in plasma, assessed using enzyme-linked immunosorbent assay (ELISA), differ significantly from those in a control population or track with progression in HD.

V.2 Contributions and collaborations

The NfH ELISA was performed by Dr Axel Petzold. Subject recruitment, plasma processing, data analysis and interpretation and manuscript preparation were carried out by the author.

V.3 Subjects and methods

V.3.1 Ethical approval and subject recruitment

Ethical approval and subject recruitment were as specified in Chapter II.

V.3.2 Inclusion and exclusion criteria

Subjects with concomitant central nervous system disorders, significant medical comorbidity, known liver dysfunction, recent alcohol or substance abuse, and those taking medications or supplements suspected or known to interfere with the experimental methods used, were excluded. In view of the likelihood of nutritional, infective and inflammatory disorders in advanced HD, such patients were not included in the study.

V.3.3 Collection and fractionation of blood samples

Subjects were classified as controls, premanifest HD or early or moderate manifest HD. Sample collection, fractionation and storage and CAG repeat sizing were carried out as outlined in Chapter II.

Plasma NfH levels were determined using an in-house ELISA technique based on commercially available antibodies (Petzold *et al.* 2003). This ELISA has been optimised for the capture antibody SMI35 (Sternberger Monoclonals Inc., Lutherville, USA) which recognizes a range of NfH phosphoforms (170 kDa, pI 6.2 to 210 kDa, pI 5.1). The analytical sensitivity of the assay was 200 pg/mL with an intra-assay variation of as little as 2.4% (Brettschneider *et al.* 2006). Values below 200 pg/mL, but above the blank reading, were interpolated from the standard curve. Values which equalled the blank reading were defined as non-measurable (0 pg/mL).

V.4 Results

Subject group	Subject number (F:M)	Median age (range)	Mean NfH concentration ± SD (pg/mL)
Control	29 (16:13)	43 (21-68)	341 ± 142
Premanifest	29 (16:13)	41 (26-53)	343 ± 180
Early	29 (15:14)	49 (25-66)	326 ± 177
Moderate	30 (21:9)	52 (25-77)	398 ± 212

Table 14 Demographic characteristics of subjects and mean plasma NfH concentrations

(Raw NfH data supplied by A Petzold.)

The demographic characteristics of subjects whose plasma was tested are shown in Table 1. The mean coefficient of variation of the assay for this experiment was 2.72. Mean NfH plasma concentrations are given in Table 1 and shown in Fig. 1. Though the mean plasma NfH level was higher in the moderate disease group, linear regression revealed no overall trend in NfH levels with advancing disease (p=0.2917) even when correcting for subject age (p=0.5423). Analysis of inter-group differences using ANOVA revealed no significant differences between subject groups (p=0.4317).

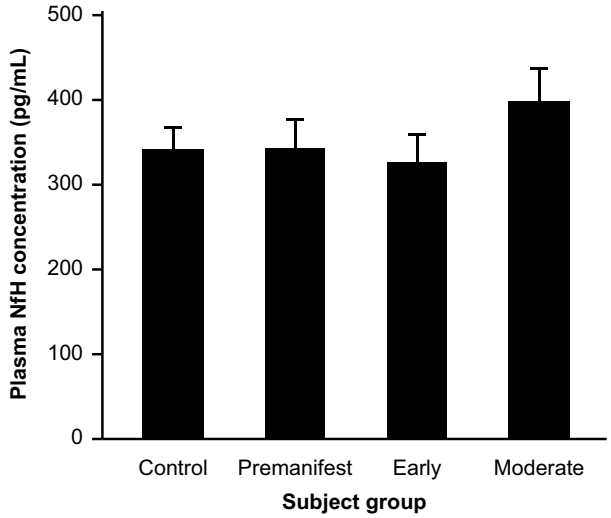

Figure 37 Plasma NfH levels are unaltered in HD

Mean values are shown in pg/mL with standard error bars. Though NfH levels were higher in moderate disease than other groups, the difference was not statistically significant and there was no significant correlation between disease stage and plasma NfH concentration. (Raw NfH data supplied by A Petzold.)

There were no significant correlations between plasma NfH concentration and either calculated 5-year probability of disease onset (Langbehn *et al.* 2004) in premanifest subjects (R^2=0.0064, p=0.6850), or disease severity (Penney *et al.* 1997) in all mutation carriers (given by [CAG repeat length - 35.5] × age; R^2=0.0044, p=0.5557).

To examine whether plasma NfH levels reflect rate of progression in HD, an analysis of genotype-phenotype discordance was carried out by identifying two subsets of mutation carriers. From their CAG repeat length, the age at which each subject would have a 50% probability of disease onset was calculated (Langbehn *et al.* 2004). The 'late-onset' group was defined as those premanifest subjects who had remained disease-free beyond this 50% probability-of-onset age (6 of 29 subjects), while the 'early-onset' group was defined as those subjects who developed manifest disease before the 50% probability-of-onset age (13 of 57 subjects). Plasma NfH levels (means ± SD, ng/L) were 0.336 ± 0.159 in the 'late onset' group and 0.380 ± 0.139 in the 'early onset' group, with no significant difference between the groups (t-test, p=0.5502).

V.5 Discussion

Using a highly sensitive ELISA assay, plasma concentrations of NfH were quantified in control subjects and HD mutation carriers from premanifest, early and moderate disease stages. Plasma NfH levels can be quantified in a robust and reproducible manner in these patients. However, a correlation between disease stage and plasma NfH level was not found, even when correcting for subject age. There was no significant correlation between plasma NfH and calculated parameters based on CAG repeat length, the major determinant of disease course in HD. Finally, no effect of genotype-phenotype discordance on plasma NfH levels was found to suggest that NfH may be a predictor of disease onset.

These findings may be explained by the speed of disease progression in HD patients. Neurofilament proteins are released from degenerating neurons and axons into the extracellular fluid, from where they equilibrate with other fluids such as blood. Because the neurodegenerative process in HD is slow, it is likely that neurofilament proteins released into plasma will be removed too quickly to allow for a measurable increase compared with controls.

Overall, plasma NfH does not fulfil the characteristics of a useful biomarker of onset or progression in HD.

V.6 Publication relating to this chapter

The work in this chapter was published as Wild EJ *et al*. (2007) *Plasma neurofilament heavy chain levels in Huntington's disease*. **Neuroscience Letters** 417(3): 231-233. Figure 37 is reproduced from this article by permission of Elsevier.

Chapter VI Whole-brain atrophy as a biomarker of HD

VI.1 Introduction

THIS CHAPTER DESCRIBES the main outcome of the longitudinal imaging study: the measurement of whole-brain atrophy over two years in premanifest and early HD. HD usually begins insidiously in mid-adult life and progresses relentlessly, causing widespread impairment of brain function resulting in an unpredictable spectrum of clinical manifestations including movement disorders, cognitive impairment and psychiatric disturbances.

The mutant huntingtin protein is expressed ubiquitously in both CNS and peripheral tissue (Aronin *et al.* 1995; Sathasivam *et al.* 1999; Björkqvist *et al.* 2008). Both post-mortem and *in vivo,* the most striking macroscopic change in HD brains is atrophy of the neostriatum with a preponderance for the caudate and putamen (Forno *et al.* 1979; Oliva *et al.* 1993). Nuclear and cytoplasmic aggregates of mutant huntingtin — the microscopic neuropathologic hallmark of HD — are found widely in gray and white matter but are relatively sparse in the striatum where degeneration is most prominent (Gutekunst *et al.* 1999). Moreover, the total loss of brain mass by end-stage HD is considerably greater than could be accounted for by striatal atrophy alone (Forno *et al.* 1979; Aylward *et al.* 2004; Henley *et al.* 2006) and numerous studies have confirmed that, notwithstanding the clear early prominence of striatal involvement, HD ultimately affects the whole brain (Rosas *et al.* 2003; Rosas *et al.* 2005).

Though there are no current treatments that slow the progression of HD in humans, several putative treatments have demonstrated a disease-modifying effect in animal models (Handley *et al.* 2006), and it has been further demonstrated that reversible neuronal dysfunction precedes cell death in HD (Yamamoto *et al.* 2000). A genetic test can reliably predict those people destined to develop the disease, and in such individuals, the slowing of pathology would be expected to delay the onset of disease symptoms. Because the disease progresses slowly and is heterogeneous, and because of the limitations of clinical rating scales, trials of possible disease-modifying treatments in humans are likely to be greatly facilitated by the availability of a panel of state biomarkers that can be used to track progression of the disease reliably and distinguish between symptomatic and disease-modifying effects (see Chapter I).

Numerous neuroimaging techniques for detecting changes in HD brains have been employed, with different abilities to capture aspects of the diverse effects of the mutant protein (reviewed in chapter I.2.2). Quantifying change in structures with well-defined anatomical boundaries may be more reproducible than measuring the volumes of less easily-defined regions. More importantly, perhaps, whole-brain atrophy would be expected to capture the totality of mutant huntingtin's effects on brain volume, and is therefore less likely to overlook clinically important changes that may have significance for our understanding of either natural history or the effects of putative therapeutic agents. The brain boundary shift integral (BBSI), a semi-automated technique for quantifying whole-brain atrophy (Freeborough *et al.* 1997) has improved reliability over manual measures (Schott *et al.* 2005) and has been employed as an outcome measure in Alzheimer's disease clinical trials (Fox *et al.* 2005).

Whole-brain atrophy rates measured over a six-month period using the BBSI have previously been shown to be increased in early HD (Henley *et al.* 2006). Larger cohorts and longer inter-scan intervals are expected to produce stronger signal-to-noise ratios in disease-related measures, enabling more robust estimation of atrophy rates in HD. In addition, the study of mutation carriers prior to the diagnosis of manifest disease may reveal important early changes in the HD. Furthermore it is not known whether whole-brain atrophy proceeds at a constant rate in HD or whether it accelerates or decelerates, and to date this aspect of the disease has only been studied to a limited extent for regional atrophy (Aylward *et al.* 2000; Aylward *et al.* 2004), but has obvious implications for both our understanding of the disease and the conduct of future clinical trials involving putative disease-modifying therapies.

A large cohort of patients with HD, premanifest mutation carriers and control subjects over two years was studied prospectively, with clinical assessment and volumetric MRI scans at baseline, and one and two years later. The BBSI technique was used to compare whole-brain atrophy between different disease stages, and used atrophy rates within subjects over consecutive intervals to determine whether atrophy accelerates in HD.

VI.2 Contributions and collaborations

The study was conceived by Dr N Fox and Dr S Tabrizi and the study protocol was designed by them with Ms S Henley and the author. Subject recruitment was shared equally between the author

and Ms S Henley. MRI scanning was overseen by Dr D MacManus. Whole-brain segmentations and were carried out equally by Ms S Henley, Ms N Hobbs and the author. Dr Chris Frost gave statistical advice. All other work, including clinical and behavioural characterisation of subjects, BSI optimisation, data analysis and interpretation and preparation of figures, was carried out by the author.

VI.3 Ethical approval

Ethical approval and overall study conduct were as described in chapter II.2, page 73.

VI.4 Subjects

Whole-brain atrophy rates were calculated for 70 subjects: 19 control subjects, 20 premanifest mutation carriers and 31 early HD patients. Demographic characteristics are given in Table 15. Controls were partners or spouses of mutation carriers, or individuals previously at risk of HD with a negative genetic test for the mutation. Premanifest HD was defined as a diagnostic confidence score of <4 on the Unified Huntington's Disease Rating Scale (UHDRS) (The Huntington Study Group 1996); early HD as a diagnostic confidence score of 4 with a UHDRS total functional capacity score of 7 or more (Shoulson 1981). No subjects were taking any medication known or suspected to influence brain volume and subjects with concomitant neurological illnesses were excluded.

VI.5 Methods

VI.5.1 Imaging

VI.5.1.1 Overview

Figure 38 gives an overview of the steps from scan acquisition to calculation of the BBSI.

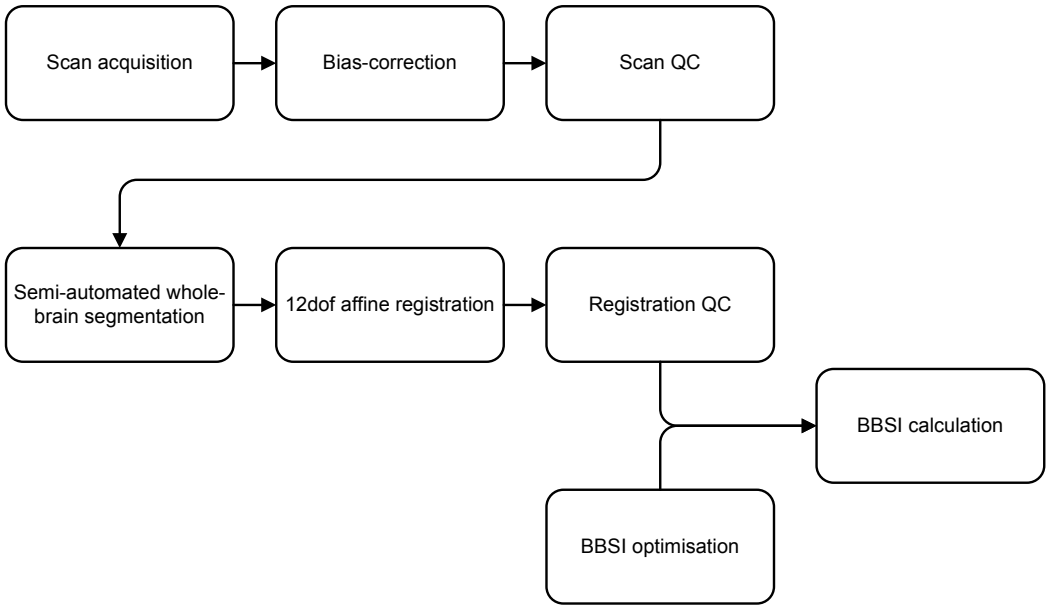

Figure 38 Overview of image acquisition, preprocessing and analysis

VI.5.1.2 Scan acquisition and preprocessing

1.5T volumetric MRI scans were acquired and preprocessed as described in chapter II.7.

VI.5.1.3 Whole-brain segmentation

Whole-brain segmentation was performed using a semi-automated algorithm (Freeborough *et al.* 1997) described in detail in Appendix H. Briefly, upper and lower thresholds were determined visually, to include all brain tissue and exclude as much non-brain tissue as possible. An axial cut-off slice was defined at the most caudal extent of the cerebellum. The region containing the brain was defined as the largest contiguous region. The region was eroded throughout by one or more voxels (in practice one erosion only was needed) to restrict the region to brain tissue. Remaining connections with non-brain tissue were deleted manually. The region was then dilated to the edge of the brain using intensity thresholds of 60% and 160% of the mean value. Where necessary, regions were manually edited for anatomical accuracy. Segmentations were performed by three investigators (including the author) whose intra- and inter-rater reliability was greater than 99%.

VI.5.1.4 Registration of serial MRI

Scan pairs were aligned using affine registration with 12 degrees of freedom, from which the BBSI was calculated (Freeborough *et al.* 1997; Woods *et al.* 1998). Registrations were carried out across

all three intervals from three timepoints: year one to baseline, year two to baseline and year two to year one.

VI.5.1.5 Optimisation of BBSI parameters

The BBSI algorithm uses values of upper and lower intensity thresholds, I_1 and I_2 (also expressed as the window centre, $I_c = I_1/2 + I_2/2$, and width, $I_w = I_1 - I_2$) which are used to determine the range of intensities over which the boundary shift is calculated, and should be selected such that it falls entirely within all of the intensity transitions (e.g. grey matter to CSF) associated with the boundaries of a structure. Adjustment of the parameters can alter the calculated BBSI substantially, while the optimum set of parameters depends largely on scan acquisition parameters, as well as on properties of the boundaries in question (Freeborough *et al.* 1997). A novel optimisation procedure developed by the author was therefore carried out to identify the parameters most likely to maximise the signal-to-noise ratio for the measure in this cohort over the longest interval studied.

Optimisation took as its gold standard the manual measure of volume change derived from the subtraction of followup from baseline brain volumes. While this measure is typically less reproducible than the optimised BBSI, the mean volume changes resulting when it is applied to cohorts of scans tend to be accurate. The optimum set of parameters was defined as those which, when applied to a representative set of registered scan pairs, produced the set of differences between the manual and BBSI-derived measure with the lowest standard deviation.

A subset of scans may be used to determine the optimum parameters for a larger set (Freeborough *et al.* 1997). Five scan pairs were selected in each subject group, such the subset matched as closely as possible the entire group. Scans were matched for mean and range of age, sex, CAG (in the premanifest and HD groups) and BBSI-derived atrophy rates using the default parameters. Because the BBSI operates on affine-registered scan pairs, it was necessary for the volume-subtraction measure to be performed on registered scans too. The whole-brain regions of the registered followup scans for these fifteen subjects were therefore re-segmented and volume changes were calculated from these re-segmented registered pairs.

To identify the optimum combination of parameters, a script written in Microsoft VBA was used to generate BBSI commands using large numbers of different combinations of parameters. Pilot work

had shown that the default BBSI parameters of $I_c = 0.5$ and $I_w = 0.5$ tended to be rather higher than the optimum values. Accordingly, a range of values for window centre from 0.3 to 0.6 in increments of 0.01 was used, with a range of values for window width from 0.1 to 0.5 in increments of 0.05. Each possible combination of centre and width was tested, for a total of 279 combinations. The outcome of the comparisons is shown in Figure 39.

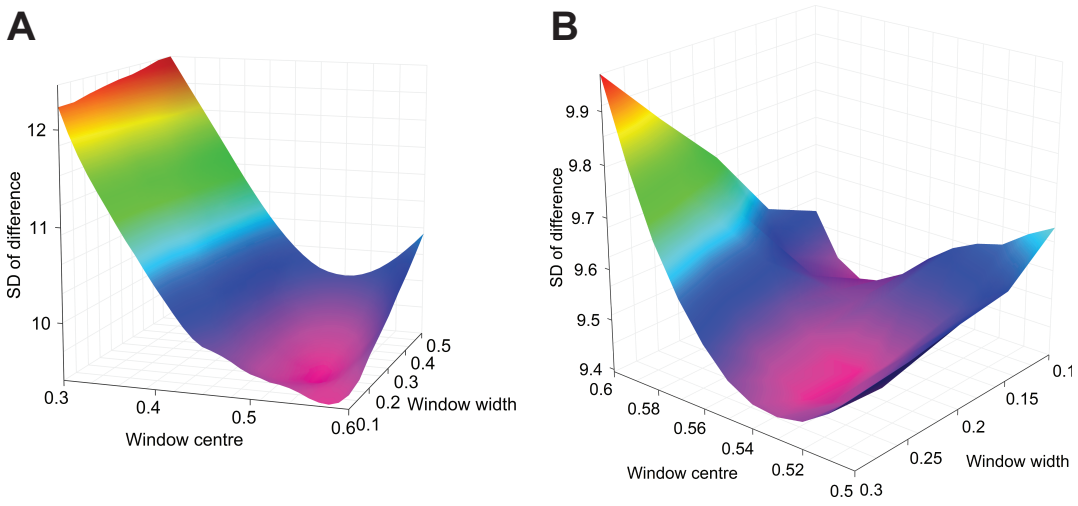

Figure 39 Outcome of BBSI optimisation process

Surface plots showing, for combinations of window centre and width, the standard deviation of the total difference between the volume changes over two years derived from the BBSI and manual subtraction for fifteen subjects. The height and colour of the surface represents the SD. Lower SDs correspond to better agreement between the measures. ***A****. Entire range of values tested.* ***B****. Narrower range of parameters containing the lowest SDs. Plots were prepared using Graphis software (Kylebank, Inc).*

Two clusters of parameters resulted in low SDs of the difference between measures: one around $I_c = 0.57$, $I_w = 0.1$ and another around $I_c = 0.55$, $I_w = 0.2$. Overall, the latter cluster contained a larger number of combinations with low SDs, in addition to having a window width less likely to miss important features that may not have been represented in the subset of scans chosen for optimisation. The final parameters chosen were therefore $I_c = 0.55$ and $I_w = 0.2$. These parameters were used to calculate atrophy rates using the BBSI over all intervals.

VI.5.2 Clinical assessment

Clinical assessment, including neurological, behavioural and functional measures, was as specified in chapter II.5-II.6. In addition subjects underwent cognitive assessment as outlined in chapter II.10 and Appendix H.

VI.5.3 Statistical analysis

VI.5.3.1 Correction for total intracranial volume

To correct for differences in head size, brain volumes were standardised for baseline total intracranial volume (TIV) which was measured according to a standard protocol (Whitwell *et al.* 2001). To derive the slope of the relationship between TIV and brain volume, log-transformed brain volumes were regressed on log-transformed TIVs for control scans. The slope, β, was then used to adjust all baseline brain volumes for TIV using the following equation:

$$V_{adjusted} = V_{raw} \times \left(\frac{Mean\,TIV}{Subject\,TIV} \right) \times \beta$$

Followup brain volumes were calculated from baseline brain volume and BBSI-derived absolute brain loss. All brain volumes were normalized to mean TIV.

VI.5.3.2 Atrophy rate calculation

Brain volume change measured by the BBSI was also converted to percentage of baseline brain volume and annualized to generate atrophy rates. All statistical analyses were carried out on log-transformed values as described in chapter II.11.13.1. Results were converted back to annualised percentage rates.

VI.5.3.3 Inter-group comparisons

Linear regression models, adjusting for age at baseline and gender, were used to compare atrophy rates acceleration between groups. Separate linear regression models of the effects of age and sex across all subjects were used to produce adjusted values for atrophy rate and acceleration.

VI.5.3.4 Clinical associations

Linear regression models, adjusting for age at baseline and gender, were used to assess associations between atrophy parameters and clinical variables.

VI.5.3.5 Genetic associations

Five-year conditional onset probabilities, and estimated time to a 60% probability of motor diagnosis were calculated using a standard formula (Langbehn *et al.* 2004).

VI.5.3.6 Sample size calculations

Sample size requirements for clinical trials were calculated using a standard approach (Campbell *et al.* 1993) based on estimating the subject numbers required to achieve a 90% chance of detecting a 30% reduction in both the absolute atrophy rate in early HD and the excess of atrophy in early HD with respect to the control rate, at a significance level of 0.05. Sample sizes were compared using atrophy rates from baseline to year one, and from baseline to year two.

VI.5.3.7 Linear change over two years

For the 'direct' measures of change (clinical, behavioural and functional scores), a generalised estimating equation (GEE) analysis was used to incorporate all available data over the three timepoints, as described in chapter II.11.13.2. The BSI, an 'indirect' measure of change, was analysed using linear regression over the longest available interval, from baseline to two years.

VI.5.3.8 Linearity of progression

To examine non-linearity within each group, paired t-tests within groups were used to compare rates of change in each variable of interest between the first and second year. For inter-group comparisons of non-linearity, difference scores were calculated by subtracting the first- from the second-year annualized rate of change. A linear regression model was then used to compare these difference scores between groups, adjusting for age and sex.

VI.6 Results

VI.6.1 Subject disposition

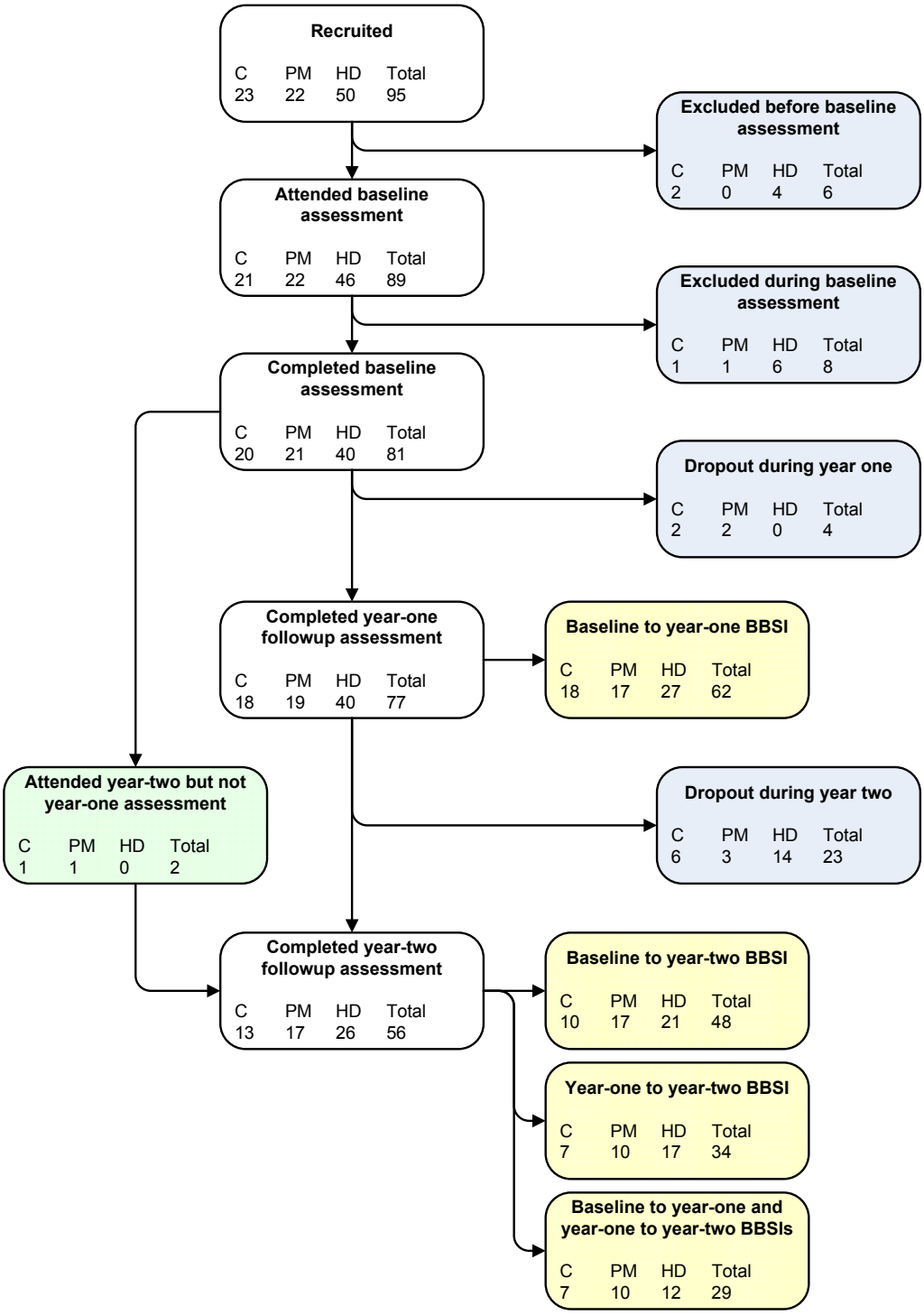

Figure 40 Subject disposition over the course of the study

C, control; PM, premanifest; HD, early HD.

As shown in Figure 40, a total of 95 subjects initially agreed to participate in the study. Six subjects did not attend the initial assessment, and of the 89 that did, eight were excluded after failing to meet the inclusion criteria when assessed in person, leaving a definitive cohort size of 81 subjects with a complete baseline assessment. During the first year of followup, four subjects dropped out (a retention rate of 95%) leaving 77 with two full assessments. Of these scan pairs, 62 produced good-quality registrations for calculation of the 12-month BBSI. During the second followup interval, a further 23 subjects dropped out, but two who missed the one-year assessment returned, so 56 subjects were assessed at the year-two timepoint. This final timepoint generated 48 successful scan registrations from baseline to two years, and 34 from one to two years. Analysis of atrophy acceleration requires registrations over both one-year intervals to be successful: this was the case for 29 subjects.

VI.6.2 Demographic data

Demographic data are given in Table 15 for the entire cohort at baseline. In addition, the sections below give separate demographic data for the subset of subjects undergoing each analysis. Overall, the groups were well-matched for IQ (t-tests), gender and handedness (Fisher's exact tests). The premanifest group was significantly younger than both controls and early HD (t-test, p=0.01 and p<0.001 respectively), but age was corrected for statistically in all analyses. The early HD group had significantly higher CAG repeat lengths than did the premanifest group (p=0.01).

	Control	Premanifest	Early HD
N	20	21	40
Gender, M:F	7:13	10:11	11:20
Age at baseline assessment, years	45.3 (10.5)	37.7 (7.9)	49.0 (9.6)
CAG repeat length	-	42.2 (1.8)	43.7 (2.4)
Years to predicted motor onset (Langbehn *et al.* 2004)	-	17.7 (7.1)	-
Disease duration	-	-	4.6 (2.7)
Total functional capacity	13 (0)	13 (0)	10.9 (1.8)
Handedness, R:L	19:1	20:1	36:4
IQ	106 (11.6)	103 (9.2)	105 (13.0)
UHDRS motor score	1.1 (0.9)	3.6 (4.0)	28.9 (12.6)
Beck depression inventory score	5.55 (3.9)	6.8 (6.3)	8.35 (8.7)
SBA score	7.1 (5.7)	8.9 (6.1)	10.5 (7.2)

Table 15 Subject demographic characteristics for the entire 81-subject imaging cohort at the baseline assessment

VI.6.3 Clinical progression

Clinical and behavioural scores at baseline are discussed in Chapter VII in the context of the VBM analysis of associations with regional atrophy. This section examines the longitudinal trends in clinical, behavioural and functional scores over the full course of the study, using a generalised estimating equation analysis. The analysis of clinical progression employs the whole cohort as described in Table 15. The results are given in Table 16. In early HD, UHDRS motor score increased by a mean of 3.9 points per year and TFC declined at a mean of 0.54 points (both $p<0.01$) but neither measure changed significantly in controls or premanifest HD subjects. Scores on the Beck depression inventory (BDI) worsened significantly in controls (1.64 points per year, $p<0.01$) and early HD (1.70 points per year, $p=0.02$) but not premanifest subjects; the difference between controls and early HD was not statistically significant however. There were no significant changes or intergroup differences (over one year only) in mean SBA score.

	Rates of change, points per year (95% confidence intervals)		
	Control	**Premanifest**	**Early HD**
N	20	21	40
UHDRS motor score (all subjects)	0.01 (-0.34 to 0.37) p=0.95	0.40 (-0.41 to 1.23) p=0.33	2.79 (1.50 to 4.08) p<0.01[a,b]
UHDRS motor score (single rater)	0.17 (-0.23 to 0.62) p=0.46	0.46 (-0.45 to 1.37) p=0.32	3.90 (2.71 to 5.08) p<0.01[a,b]
TFC	-0.02 (-0.09 to 0.05) p=0.53	-0.02 (-0.08 to 0.04) p=0.58	-0.54 (-0.80 to -0.29) p<0.01[a,b]
Beck score	1.64 (0.62 to 2.67) p<0.01	-0.49 (-1.42 to 0.44) p=0.32[a]	1.71 (0.27 to 3.15) p=0.02[b]
SBA score*	0.22 (-2.69 to 3.13) p=0.88	-1.43 (-4.48 to 1.62) p=0.36	1.49 (-0.80 to 3.77) p=0.20

Table 16 Annual rates of change of clinical variables.

GEE analysis is adjusted for age, gender and, for UHDRS motor score in all subjects, motor rater. Mean rates are given with 95% CIs. p *values indicate whether this change was significantly different from zero. The superscripts indicate where the change per year differed between groups: [a]change different from that in controls; [b]change different from that in premanifest HD (both at* p<*0.05). *SBA scores at the two-year timepoint were not included because of a change in the assessment tool (see chapter II.6).*

Assessment of linearity of change in clinical scores, not including SBA, was carried out in the 56 subjects who attended all three assessments. Their demographic data and the results of the analysis of non-linearity by paired t-test within groups are given in Table 17.

There appeared to be a significant slowing of progression of motor score in the premanifest group (year one change +2.0; year two change -0.4; p=0.03). However, when the intra-group comparison was limited to those subjects assessed by the principal motor rater, the change was not significant (year one change +1.7, year two change -0.1; p=0.19) and when analysed in the entire premanifest group with the effect of motor rater adjusted for statistically, again there was no evidence of significant change in the rate of progression (p=0.47).

For the other clinical variables, there was no evidence of significant non-linearity within any subject group, or when compared between groups (data not shown), and non-linearity scores were not significantly associated with baseline disease duration, TFC, motor score, conditional onset probability or CAG repeat length (after adjustment for age).

		Control		Premanifest		Early HD	
N		12		16		28	
Gender, M:F		5:7		8:8		13:15	
Age at baseline assessment, years		45.3 (7.4)		39.1 (8.1)		48.3 (10.0)	
CAG repeat length		-		41.9 (1.6)		43.4 (2.3)	
Years to predicted motor onset at baseline (Langbehn *et al.* 2004)		-		15.7 (7.2)		-	
Disease duration, years		-		-		4.9 (2.7)	
Change in clinical scores:	**UHDRS motor (all subjects)**	0.9 -0.8	(2.4) (1.5) *p*=0.13	2.0 -0.4	(3.7) (1.4) ***p*=0.03**	4.8 1.1	(7.4) (8.1) *p*=0.13
year 1 (SD) year 2 (SD) *p*-value for non-linearity	**UHDRS motor (single rater)**	2.2 -1.5	(3.9) (2.5) *p*=0.33	1.7 -0.1	(3.9) (1.4) *p*=0.19	4.8 3.5	(7.9) (6.8) *p*=0.64
	TFC	0 -0.1	(0) (0.2) *p*=0.33	0 0	(0) (0.2) *p*=0.33	-0.6 -0.7	(1.1) (1.1) *p*=0.88
	BDI	2.1 1.3	(5.5) (4.2) *p*=0.88	0.7 -1.0	(4.6) (5.1) *p*=0.77	-0.6 2.7	(6.6) (6.2) *p*=0.46

Table 17 Demographic data and outcomes of intra-group tests for non-linearity in rates of change of clinical scores.

Groups were well-matched for gender. The premanifest group was significantly younger than controls and early HD and had significantly shorter CAG repeat lengths than the early HD group.

VI.6.4 Whole-brain atrophy rates over two years

VI.6.4.1 Overview

In order to present the whole-brain outcomes visually for as much of the cohort as possible, TIV-corrected baseline brain volumes and subsequent atrophy values were used to generate TIV-corrected followup brain volumes, which are shown in Figure 41 for all subjects for whom a

minimum dataset of a baseline TIV and at least one value for whole-brain atrophy (from baseline to years one or two) was available. The demographic data of these subjects are given in Table 18.

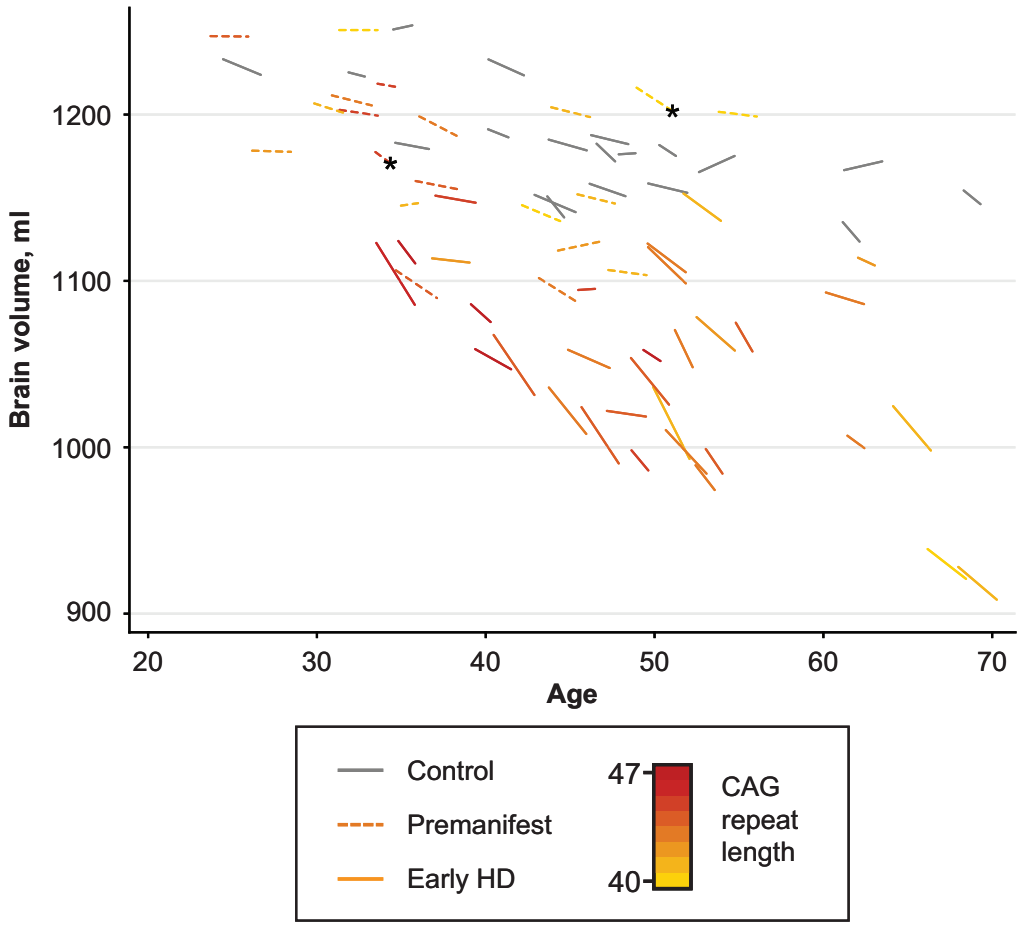

Figure 41 Whole brain MRI findings in this cohort

All brain volumes (vertical axis) are normalized to TIV. Each subject's baseline brain volume is joined to the BBSI-derived followup brain volume over the longest assessment interval. In HD gene carriers, line colour represents the pathological CAG repeat length. In controls (grey), a slow decline in brain volume reflects normal age-related atrophy, and the slopes (atrophy rates) are largely within the range expected for such age-related atrophy. Premanifest carriers generally lie within the control range for brain volume and atrophy rate, but several lie outside the control range for one or both measures. Two premanifest subjects who underwent motor onset during the study are highlighted with asterisks at the point of onset diagnosis. In early manifest HD, brain volumes and atrophy rates generally lie outside the control range, with higher CAG repeat length subjects tending to undergo pathological atrophy earlier and to a greater extent.

	Control	Premanifest	Early HD
N	19	20	31
Gender, M:F	7:12	9:11	16:15
Age at baseline assessment, years	45.6 (10.7)	37.5 (8.1)	49.4 (9.1)
CAG repeat length	-	42.3 (1.8)	43.4 (1.7)
Years to predicted motor onset (Langbehn *et al.* 2004)	-	15.6	
Disease duration	-	-	4.9 (2.7)
Total functional capacity	13 (0)	13 (0)	11.2 (1.7)
UHDRS motor score	1.1 (0.9)	3.6 (4.1)	27.6 (11.7)
Interval between baseline and 1-year scan, years	1.0 (0.1)	1.0 (0.1)	1.0 (0.1)
Interval between baseline and 2-year scan, years	2.2 (0.1)	2.3 (0.1)	2.3 (0.1)

Table 18 Baseline demographic data for the subjects whose whole-brain atrophy findings are shown in Figure 41

*The groups were well-matched for gender. The premanifest group was significantly younger than both controls (*p=*0.01) and the early HD group (*p<*0.001) and had significantly lower CAG repeat lengths (*p=*0.02). There were no significant inter-group differences in inter-scan duration for either interval.*

VI.6.4.2 Improved signal-to-noise ratio

Overall the quality of scan registrations was higher over the two-year period than over one year, and the BBSI technique appeared to be better able to capture true atrophy over the period. Examples of whole-brain atrophy in an HD patient and a control subject measured by the BBSI over one and two years, highlighting both the disease-related increase in atrophy and the improved detection of atrophy over two years, are given in Figure 42.

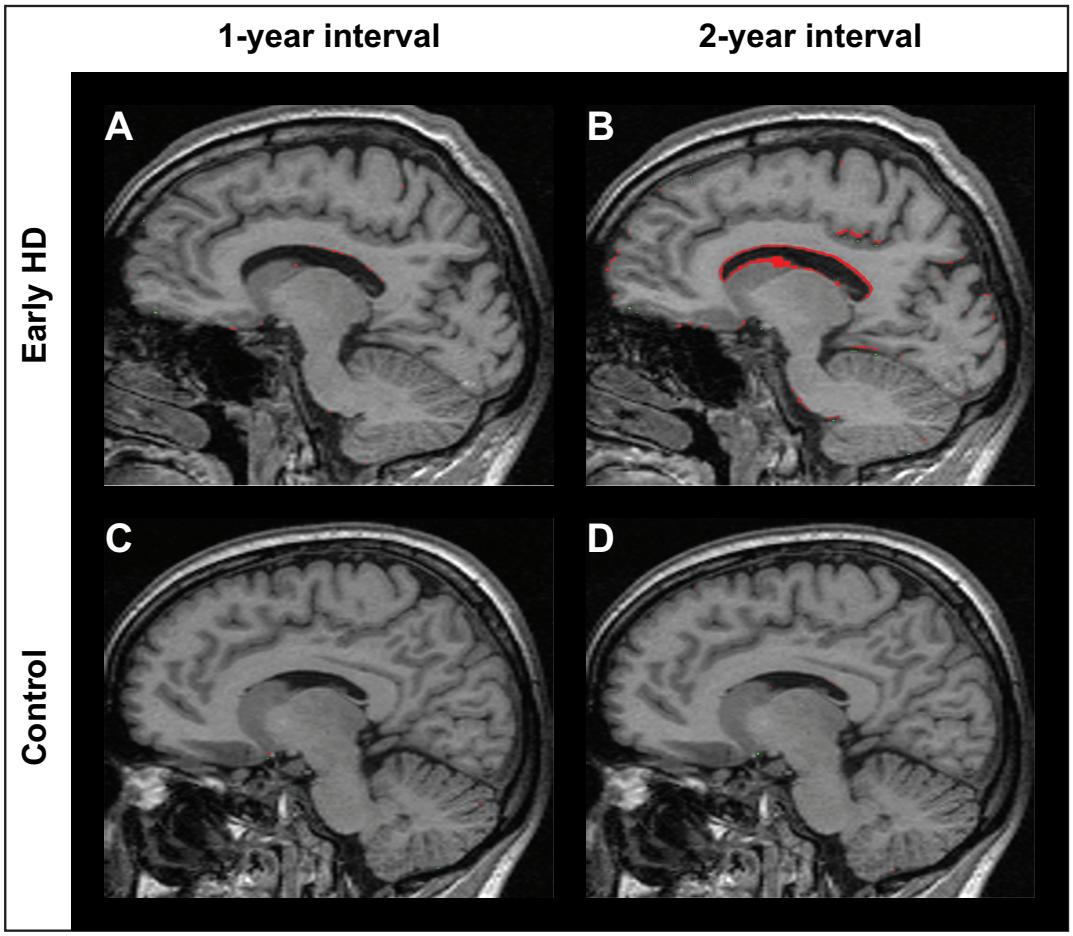

Figure 42 Whole-brain atrophy in HD quantified by the BBSI technique

Parasagittal views of baseline MRI scans with overlays showing areas of volume loss (red) and gain (green) detected by BBSI. ***A****. Early HD patient aged 33 with 47 CAG repeats, showing change over one year.* ***B****. Change over two years in the same patient, with clearer evidence of atrophy. C-D. Equivalent images from a 40-year old control subject, showing little atrophy over either interval.*

VI.6.4.3 Atrophy rates

Whole-brain atrophy rates over two years were calculated for 48 subjects, whose demographic data are given in Table 19.

	Control	Premanifest	Early HD
N	10	17	21
Gender, M:F	6:4	8:9	11:10
Age at baseline assessment, years	44.2 (10.0)	38.2 (8.7)	48.5 (9.7)
CAG repeat length	-	42.0 (1.7)	43.1 (1.9)
Years to predicted motor onset (Langbehn *et al.* 2004)	-	16.0 (7.0)	-
Disease duration	-	-	4.8 (2.6)
Total functional capacity	13 (0)	13 (0)	11.6 (1.5)
UHDRS motor score	1.1 (0.9)	2.8 (3.2)	26.0 (10.6)
Interval between baseline and 2-year scan, years	2.2 (0.1)	2.3 (0.1)	2.3 (0.1)

Table 19 Baseline demographic data for the 48 subjects whose whole-brain atrophy rates were calculated over the two-year interval

*The groups were well-matched for gender. The premanifest group was significantly younger than the early HD group (*p<*0.01) but not controls (*p=*0.11). The two gene-positive groups did not differ significantly in CAG repeat length (*p=*0.055).*

Whole-brain atrophy rates by group are given in Table 20 and Figure 43. Mean atrophy rate was about five times higher in the HD group than in both controls and the premanifest HD group and the inter-group differences were statistically significant ($p<0.001$ in both cases). In this cohort far from predicted disease onset, the mean atrophy rate in the premanifest group did not differ significantly from the control mean ($p=0.55$).

	Control	Premanifest	HD
N	10	17	21
Mean atrophy rate, % per year (SD)	0.16 (0.25)	0.22 (0.23)	0.88 (0.50)
95% CI	-0.02 to 0.34	0.10 to 0.34	0.65 to 1.10

Table 20 Whole-brain atrophy rates by group, adjusted for age and sex

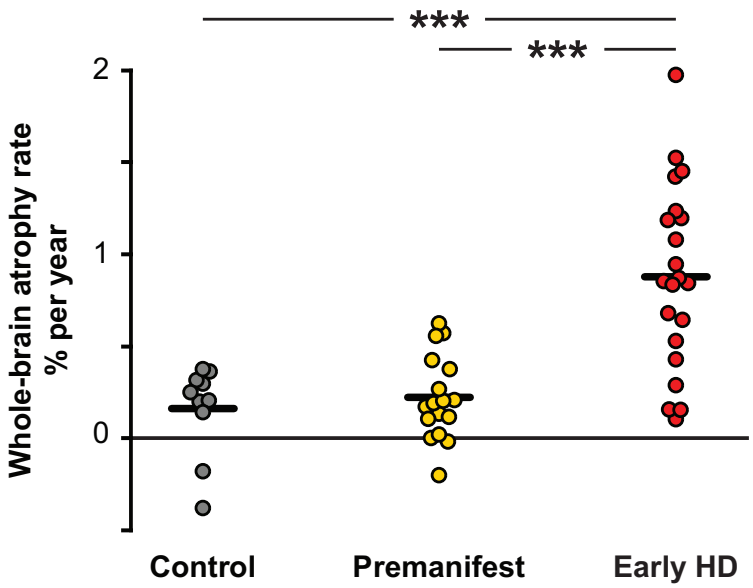

Figure 43 Whole-brain atrophy is increased in early HD

*Whole-brain atrophy rates, adjusted for age and sex, were increased in early HD patients compared with both controls and premanifest HD. ***p<0.001 by linear regression. Horizontal bars show group means.*

As shown in Figure 43, there was a statistically significant association between age- and gender-adjusted whole-brain atrophy rate and CAG repeat length (p=0.009). On average, a single triplet increase in repeat length was predicted to increase the whole-brain atrophy rate by 0.12% (95% CI 0.03% to 0.22%, p=0.009, R=0.42). As shown in Table 21, over the two-year interval, whole-brain atrophy rates were not significantly associated with baseline disease duration, onset probability in the premanifest group or change in any clinical variable, though the association with change in UHDRS chorea score was close to statistical significance (p=0.06).

Predictor	Group	R	*p*	Slope of association, % per year per unit (95% CI)
CAG repeat length	PM & HD	-0.4247	0.009	0.12% (0.03% to 0.22%)
5-year onset probability	PM	-0.2042	0.48	0.63% (-1.26% to 2.49%)
Baseline motor duration	HD	-0.2465	0.23	0.05% (-0.03% to 0.13%)
Change in UHDRS motor score[a]	HD	0.1054	0.68	-0.02% (-0.09% to 0.06%)
Change in UHDRS chorea score[a]	HD	0.3213	0.06	-0.14% (-0.28% to 0.01%)
Change in TFC[b]	HD	0.1967	NS	-0.15% (-0.45% to 0.49%)
Change in BDI score[b]	PM & HD	0.2793	NS	-0.01% (-0.11% to 0.09%)

Table 21 Associations between whole-brain atrophy and clinical variables over two years

[a] *Motor rater was used as a covariate in the analysis of associations with clinical scores.*

[b] *Bootstrapping was used to analyse these non-normally distributed variables, so a* p*-value is not obtained directly; NS, not significant at the 5% level.*

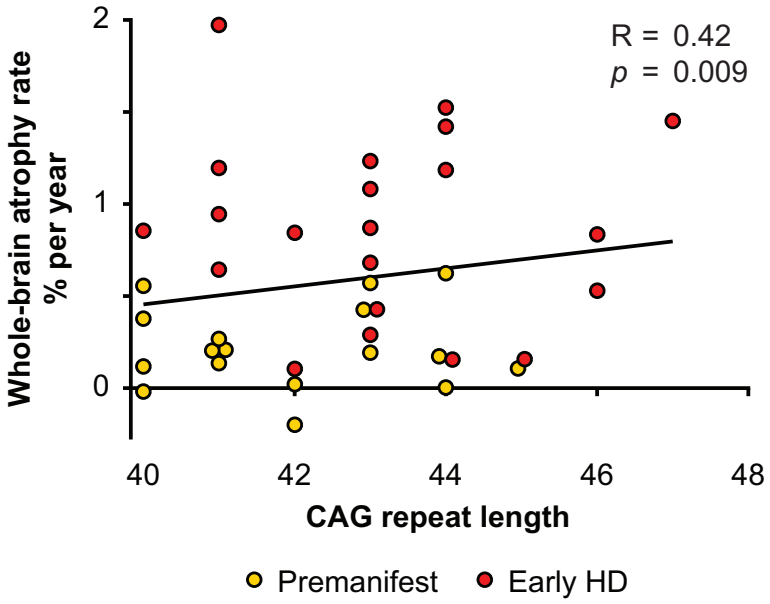

Figure 44 Association of whole-brain atrophy rate with CAG repeat length across premanifest and early HD

This relationship is partly explicable by the fact that the premanifest group had more subjects with low repeat lengths and fewer with high repeat lengths, while HD subjects have higher rates of atrophy than premanifest subjects. On average, a single triplet increase in repeat length was predicted to increase the whole-brain atrophy rate by 0.12%.

Sample size estimations were calculated for putative clinical trials using whole-brain atrophy as an outcome measure, to determine the number of early HD subjects per arm required for trials of a treatment reducing atrophy rate by 30%, both in absolute terms and with respect to the difference between early HD and controls. For a one-year trial calculated using absolute atrophy reduction, the requirement would be 120 subjects; for a two-year trial, 78 subjects. For the same trial based on relative reduction, the requirements would be 322 and 116 subjects respectively.

VI.6.4.4 Atrophy acceleration

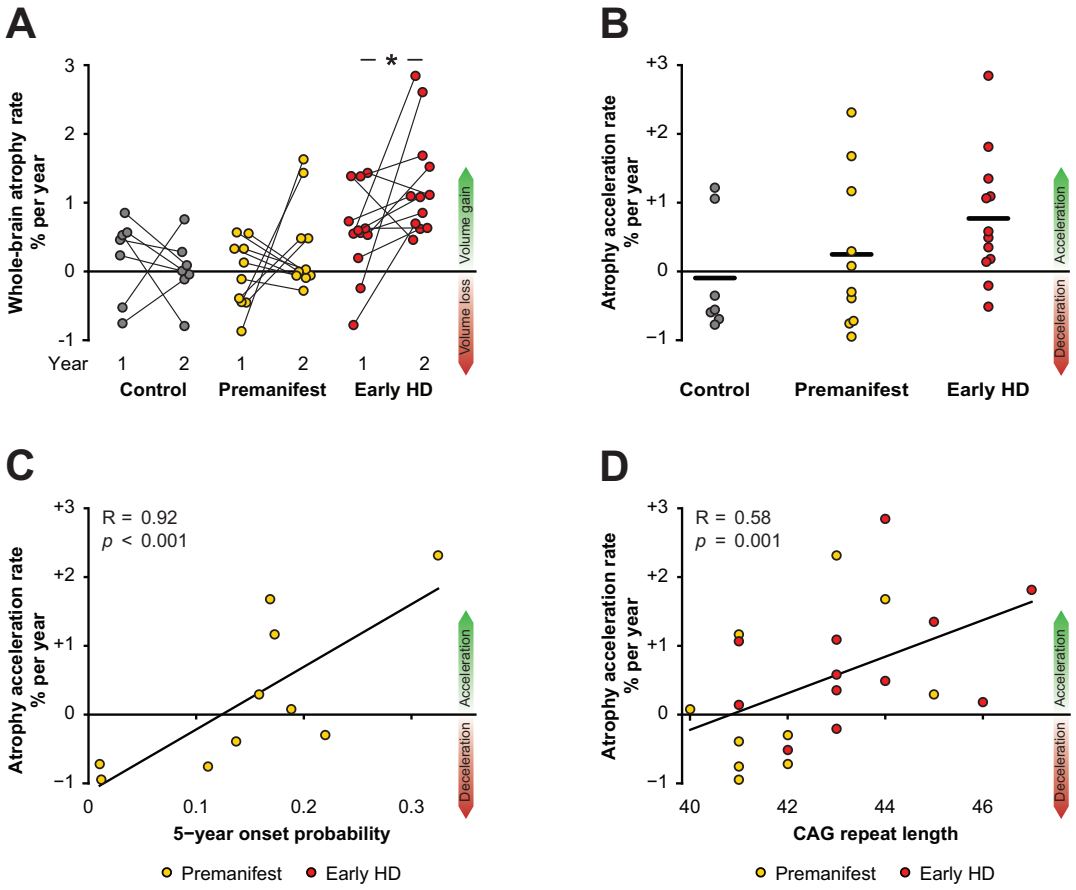

Figure 45 Whole-brain atrophy accelerates in early HD, and acceleration increases as motor onset approaches in premanifest HD and correlates with pathological CAG repeat length

A. *Whole-brain atrophy rates by group, with each subject's first- and second-year atrophy rates joined, such that accelerating atrophy is indicated by an upward slope. Atrophy rates did not increase significantly in controls and premanifest HD but did so in early disease (*p<0.05, paired t-test).* ***B***. *Age- and sex-adjusted atrophy acceleration rates, showing the difference in atrophy rate between the second and first years. Values above zero represent atrophy acceleration. Horizontal bars show group means.* ***C***. *Age- and sex-adjusted atrophy acceleration rates show a strong positive association with 5-year conditional onset probability in the premanifest group. A 10% increase in onset probability predicts a 1.4% annual acceleration in atrophy (95% CI: 1.0% to 1.8%).* ***D***. *Age- and sex-adjusted atrophy acceleration rates show a positive association with CAG repeat length: on average, a one-unit increase in CAG repeat length is associated with a predicted increase in annual atrophy acceleration of 0.4% (95% CI: 0.2% to 0.6%). R, partial correlation coefficient.*

Annualized atrophy rates were calculated for a subset of 29 subjects across the two consecutive one-year intervals (Figure 45 and Table 22). Comparison of the first and second year atrophy rates within each group (Figure 45A) revealed significant acceleration of atrophy in the early HD group (p=0.048) but not in controls or premanifest HD (p=0.38 and 0.28 respectively). The acceleration of atrophy remained statistically significant in the early HD group (p=0.045) if determined using absolute volume loss (ml per year), rather than relative loss (% per year). When acceleration was compared between groups, the HD group tended to accelerate compared with controls, though the difference was not statistically significant (p=0.055).

	Control	**PM**	**HD**
N	7	10	12
Gender, M:F	4:3	5:5	6:6
Age at baseline assessment, years	46.7 (8.7)	40.0 (8.9)	46.4 (9.1)
CAG repeat length	-	42.0 (1.6)	43.5 (1.8)
Years to predicted motor onset (Langbehn *et al.* 2004)	-	14.3 (6.8)	-
Disease duration	-	-	5.5 (3.0)
Total functional capacity	13 (0)	13 (0)	11.6 (1.7)
UHDRS motor score	0.9 (0.9)	3.1 (3.6)	27.0 (11.1)
Interval between baseline and 1-year scan, years	1.0 (0.0)	1.0 (0.0)	1.0 (0.1)
Interval between 1-year and 2-year scan, years	1.2 (0.1)	1.3 (0.1)	1.3 (0.1)
0-1 year atrophy rate (SD)	0.20 (0.60)	-0.03 (0.49)	0.58 (0.66)
1-2 year atrophy rate (SD)	0.03 (0.47)	0.36 (0.67)	1.27 (0.79)
Mean annual atrophy acceleration rate from year 1 to year 2 (SD)	-0.17 (0.88)	+0.39 (1.10)	+0.69 (1.08)
95% CI	-0.99 to +0.64	-0.40 to +1.18	+0.01 to +1.37

Table 22 Assessment of whole-brain atrophy acceleration by group.

Units are % of baseline whole brain volume per year. There were no significant inter-group differences in sex, age, CAG repeat length or inter-scan interval.

Despite the absence of evidence for an overall acceleration of atrophy in the premanifest group, there was a striking association between these subjects' 5-year onset probability and acceleration of whole-brain atrophy, such that a 10% increase in onset probability predicted a 1.4% annual acceleration in atrophy (95% CI 1.1 to 1.7 Figure 45C; p<0.001). This suggests that significant acceleration of whole-brain atrophy occurs as manifest disease onset approaches.

Across all gene carriers (Figure 45D), there was a positive association between annual acceleration of atrophy and CAG repeat length, after correction for age and sex, such that, on average, a single triplet increase was associated with a 0.4% annual acceleration in atrophy (p=0.001).

Apart from onset probability in the premanifest group and CAG repeat length in all gene carriers, atrophy acceleration was not associated with other disease features (namely baseline disease duration, TFC or baseline motor score).

VI.7 Discussion

VI.7.1 Clinical change

As expected, UHDRS motor, TFC and Beck depression inventory scores, analysed by generalised estimating equation analysis, declined significantly in early but not premanifest HD (Table 17). The rate of decline in motor scores in this cohort is slightly slower than that seen in other cohorts (e.g. The Huntington Study Group 1996), probably reflecting the early stage of the disease in these patients, as well as perhaps the availability of antichoreic medications which may affect the motor score.

The addition of a second motor rater for some subjects at the two-year timepoint (because of integration of the study with TRACK-HD) has some interesting consequences. To an extent, the effect of inter-rater variability can be corrected for by using the motor rater as a covariate (as in Table 16). However, if the rates of progression are analysed excluding subjects assessed by the second rater, the values are rather different (3.9 points/year decline in early HD, as opposed to 2.8). This highlights a shortcoming of the generalised linear model: it can only model and correct for effects of nuisance variables where those effects are linear between groups and across timepoints. However it is likely that the effect of motor rater differed between groups (here, the second rater appears to give disproportionately lower scores in early HD) and between timepoints (the second

rater was only involved at the year two timepoint). The differential effect of motor rater is echoed by the findings for the analysis of linearity of UHDRS motor score (Table 17), where an unexpected finding appears to arise in the premanifest group: that UHDRS motor scores rose in the first year and fell in the second, with the difference between the two changes being statistically significant. However, once the analysis is limited to those subjects scored by the primary rater, the difference is non-significant, as it is when the whole cohort is examined with motor rater as a covariate. That the UHDRS motor score varies considerably between raters is well known (The Huntington Study Group 1996) but these findings emphasise the crucial importance of consistency in motor rater, both cross-sectionally and longitudinally, for studies such as this, as well as the incomplete ability of statistical models to correct for differences between motor rater, even when these have been expected in advance, and training has been given to seek to minimise inter-rater variability.

The rate of functional decline in this early HD cohort was rather slower than that previously reported: 0.59 units per year decline, compared with typical figures of around 1 unit per year in stages I and II (The Huntington Study Group 1996; Marder *et al.* 2000). There are two likely reasons for this. Firstly, the contexts of this study — with its MRI scanning and neuropsychological assessment in addition to clinical testing — is more intensive from previous, purely clinical studies; faster-progressing subjects may be less likely to volunteer for an intensive study such as this, and more likely to drop out after fewer than three assessments. Second, to an extent it likely reflects improvements in clinical care in the past decade, especially given the multidisciplinary HD clinic setting from which the early HD patients were mostly recruited.

Interestingly, while advancing HD was associated with significant worsening in the BDI score, the early HD group did not differ significantly from controls in rate of change of BDI, because control subjects' scores worsened significantly too, at a rate comparable to that seen in early HD. As most of our controls were partners or spouses of premanifest or manifest subjects, this likely reflects the growing psychosocial burden on them, and perhaps, in addition, the reduced availability of support for carers and partners compared with patients themselves.

The short behavioural assessment could not be applied at all timepoints, but was available at baseline and one year. Rates of change of SBA were not significantly different from zero in any group over this interval, and did not differ significantly between groups. This is interesting, especially

given the suggestion (discussed in Chapter VII) that the SBA is better able to detect genuine disease-related behavioural features when applied cross-sectionally. The lack of significant change over time is likely due to a combination of factors. First, the availability of effective treatments for behavioural features of HD, which may be able to stave off progression selectively in HD, but will tend to be less available to control subjects (even though the partners of HD patients tend to have a progressive behavioural phenotype, as attested by SBA scores). Second, the broader scope of the SBA means that, while it may be better able to capture the full range of behavioural deficits in HD, it is perhaps less likely to progress linearly when examined within or between groups, since it is likely to be more variable between subjects or within a given subject over time, as different behavioural problems emerge and recede.

In contrast to whole-brain atrophy, there was no evidence of significant non-linearity in the key clinical measures over the two-year interval studied. This may reflect a true absence of linearity in the disease over this period. However, the apparent acceleration in atrophy in the early HD group, plus the association between atrophy acceleration and onset probability in the HD group, suggests that there may be an acceleration of underlying pathology that was not reflected in the clinical scores, highlighting the need for alternative measures.

VI.7.2 Whole-brain atrophy

Quantification of whole-brain atrophy over two years in this large cohort confirms that atrophy rate is significantly increased in early HD, to about 5 times the rate associated with normal aging. A significant increase in brain atrophy was not seen overall in this group of premanifest subjects but, given that they are relatively far from onset as a group (mean 17.6 years), this is not altogether surprising.

This study also confirms a significant association between whole-brain atrophy rate and CAG repeat length across both premanifest and manifest mutation carriers. That this association is present after correction for age indicates that the increased atrophy rate is not simply due to the tendency of the disease to be more severe with aging for a given repeat length. The association is in keeping with our work using other analysis techniques and the imaging, clinical and neuropathological findings of others (Penney *et al.* 1997; Rosas *et al.* 2001; Kassubek *et al.* 2004; Ravina *et al.* 2008). These

findings may be of importance for subject stratification for clinical trials, where the effect of CAG repeat length needs to be considered in addition to stratification according to clinical disease stage.

The rate of atrophy in early HD is consistent with our previous findings over shorter intervals (Henley *et al.* 2006), but in this longer study, the signal-to-noise ratio of the measure is considerably improved, probably because over longer time intervals, variability due to MRI acquisition or image analysis has relatively less impact on measured atrophy rates. This improvement in signal-to-noise ratio is highlighted by the calculated sample size requirements for clinical trials using atrophy rate as an outcome measure, which are about 35% lower over two years than one year, whether calculated using absolute rates or excess atrophy relative to controls. Dropout rates from cohort studies are unlikely to reach 35% over two years, indicating that a three-timepoint study over two years, allowing calculation of both atrophy rate and an assessment of atrophy acceleration, is practicable and may require fewer subjects at enrolment.

Post-mortem studies have revealed 10-20% loss in brain mass by end stage in HD, (Forno *et al.* 1979) equating to about 180ml for a typical brain. If the annual atrophy rate of 0.9% seen in early HD were sustained year-on-year, this volume of atrophy would be reached after about 17 years, roughly corresponding to the typical period of 15-20 years between onset and death (Roos *et al.* 1993). On the face of a single-interval measurement, it is therefore tempting to suggest that atrophy rate proceeds linearly at a fixed annual percentage rate. However, our data suggest that such assumptions of linearity of whole-brain atrophy rate may not be valid. Within-subject comparisons of atrophy rates in this cohort suggest that atrophy does accelerate in early HD (by about 0.7% of brain volume each year, albeit with wide 95% confidence intervals, from 0.01% to 1.4%), while acceleration precedes onset in premanifest HD and is not seen in controls. The fact that the rate of atrophy in the manifest group is about five times higher than that seen in the premanifest group or controls, means that there must have been acceleration in the rate of atrophy. What is unclear is whether the rate of loss, once established, it is sustained at that rate through to the final stages of disease.

These preliminary findings therefore merit further study in larger cohorts over multiple timepoints. Such studies are now underway, most notably in the form of TRACK-HD (TRACK-HD Steering Committee 2006-8).

Defining linearity of atrophy rate is not without difficulty: atrophy proceeding at the same number of millilitres each year would appear to accelerate if expressed as a percentage of brain volume, because atrophy reduces brain volume. There is no firm neuropathologic basis for deciding whether atrophy acceleration is best defined as an increase in absolute or relative atrophy rate. However, in this study, atrophy rates accelerated significantly, whether rates were expressed in terms of annual percentage loss or annual volume loss, suggesting that the whole-brain atrophy rate in early HD increases significantly year-on-year according to either definition of linearity.

The absence of measurable acceleration in the premanifest group as a whole may again be explicable by the fact that the premanifest group is generally many years from predicted onset. However, the striking correlation between acceleration of atrophy and estimated 5-year onset probability in the premanifest group strongly suggests that atrophy acceleration is seen in the years before clinical diagnosis in HD. Further work in larger premanifest cohorts with subjects closer to predicted motor onset is required to investigate whether year-on-year acceleration of whole-brain atrophy is a predictor of clinical onset.

Linearity of whole-brain atrophy rate in HD has not previously been studied, but multi-interval longitudinal measurement of other brain structures has been carried out. Aylward and colleagues found that caudate volume was stable until 11 years prior to motor onset, then atrophy of the caudate proceeded linearly at 0.24cm^3 per year in premanifest subjects, while putaminal volume was stable until 9 years before motor onset then volume loss occurred at 0.23cm^3 per year (Aylward *et al.* 2004). In previous work, caudate atrophy rates in mild and moderate HD were determined to be 4.9% and 7.2% respectively, suggesting acceleration with progressing disease. However, because of the small volume of the caudate, these percentage changes corresponded to roughly equal absolute annual rates of atrophy of 0.16 cm^3 and 0.18cm^3 respectively (Aylward *et al.* 2000). It is difficult to assess whether this represents a true acceleration of pathology, for the reasons of definition discussed above, and because cross-sectional comparison of single intervals between disease stages is a less reliable indicator of atrophy rate linearity than are serial observation of individuals over multiple intervals. Recent cross-sectional data from the large Predict study appear to confirm the findings of Aylward and colleagues (Paulsen *et al.* 2008).

In summary, quantification using the BBSI over two years confirms whole-brain atrophy rates to be increased in early HD with acceleration year-on-year, whereas the rate of atrophy is not increased overall in far-from-onset premanifest HD, but does accelerate as motor onset approaches. In HD mutation carriers, both rate and acceleration of atrophy tend to be greater at a given age for subjects with larger CAG repeat lengths. Study of whole-brain atrophy has the potential to inform our understanding of the neurobiology of HD and may provide one means of assessing the outcomes of future clinical trials of putative disease-modifying treatments.

VI.8 Publications relating to this chapter

A draft manuscript containing the work presented here is under preparation for submission.

The one-year longitudinal data for this study have been published as: Henley SMD / Wild EJ *et al*. (2009) *Whole-brain atrophy as a measure of progression in premanifest and early Huntington's disease*. **Movement Disorders**, published online ahead of print.

A discussion of the BBSI as a potential biomarker for HD is included in a review article by the author as: Wild EJ and Fox NC (2009) *Serial volumetric MRI in Parkinsonian disorders*. **Movement Disorders,** accepted for publication.

Chapter VII Clinical-neuroanatomical associations in early HD

VII.1 Introduction

THE PRIMARY FOCUS of the longitudinal imaging study was on atrophy measured summatively across the whole brain using the BBSI technique, with its accuracy, reproducibility and ability to capture the full extent of structural brain change, as a biomarker of progression in premanifest and early HD. But as has been alluded to, the study of biomarkers is at its core a study of natural history, since no measure that is not linked to pathogenesis can be considered a true biomarker, and the most useful biomarkers are likely to be those with the closest and best-understood links with the disease process. Outwardly, this is likely to be reflected by associations between biomarker levels and established clinical measures as discussed in chapter II.11.9. Lack of association between a potential biomarker and one or more established clinical measures is likely to undermine its potential utility.

The assertion of Chapter VI that HD is a whole-brain disease has the corollary that all brain atrophy is fundamentally regional, and crucially, the BBSI technique is a quantitative one with no localising ability. BBSI-derived overlays such as those shown in Figure 42 can highlight the areas where the brain boundary has moved by the greatest amount, but the atrophy underlying these shifts may be structurally remote from the boundary (Freeborough *et al.* 1997). Nonetheless, it is of interest to ask which brain regions the atrophy measured by the BBSI is most pronounced, and what the clinical associations of such regional atrophy are. Furthermore, the demonstration that extrastriatal areas undergo atrophy in HD is an insufficient argument for the adoption of whole-brain atrophy measures as biomarkers: it must be further demonstrated that such extrastriatal atrophy is of clinical relevance. Thus, study of regional as well as whole-brain atrophy is an important step in the evaluation of whole-brain atrophy measures as potential biomarkers. In addition, examining the clinical correlates of regional atrophy is of interest *per se* in enhancing our understanding of the neurobiological basis of HD.

Voxel-based morphometry (VBM) is an unbiased, automated technique for analysing regional volume changes across the whole brain (Ashburner *et al.* 2000). Unlike region-of-interest approaches, it requires no *a priori* hypotheses about regionalisation. In a sense, VBM is somewhat

analogous to the 'omics' techniques for laboratory biomarker discovery, in that it performs many comparisons on a macro-scale to drill down to specific foci of greatest disease-related difference. Like these techniques, VBM is not without its potential problems of interpretation, most notable among which are methods of accounting for multiple comparisons (its statistical parametric maps (SPMs) are essentially the outcome of thousands of pairwise t-tests, one for each voxel in the brain), variations in its technical implementation and concerns about a lack of standardisation in performing and reporting analyses (Bookstein 2001; Apostolova *et al.* 2007; Ridgway *et al.* 2008). Nonetheless, the technique and its reporting have improved over time and it has become a widely-established and well-validated technique for studying disease-related changes in brain structure, albeit with the caveat that it is perhaps most appropriately used as a tool for generating hypotheses about regionalisation that can then be tested with a directly focused approach.

As reviewed in chapter I.2.2, VBM has been applied to Huntington's disease and, though findings have not been entirely consistent between studies, most studies have confirmed the presence of striatal atrophy (Thieben *et al.* 2002; Peinemann *et al.* 2005) with a general consensus that extra-striatal areas also undergo atrophy in early HD (Kassubek *et al.* 2004; Douaud *et al.* 2006).

In addition to groupwise contrasts, comparing regional brain volumes in, say, HD patients versus controls, VBM may be used to examine associations between regional atrophy and phenotypic measures, typically clinical scores.

In one such study of the structural correlates of motor dysfunction in HD, a subgroup analysis of patients with mild and severe motor involvement revealed associations with volume in the striata, opercula and hypothalamus (Kassubek *et al.* 2004). In a more recent study, higher UHDRS motor score was shown to correlate with reduced grey-matter volume in both caudate nuclei, the right hippocampus, the calcarine fissure and the left cerebellum, with several regions also displaying decreased white-matter volume in association with motor score (Jech *et al.* 2007). In addition, cortical thickness measurement has revealed apparently extensive cortical involvement in early HD, while distinct motor phenotypes (bradykinetic and choreic) appear to be differentially associated with atrophy of different areas, with the more common hyperkinetic phenotype affecting both parietal, frontal (specifically the precentral gyri) and occipital regions (Rosas *et al.* 2008).

The regional morphometric associations of the behavioural / psychiatric phenotype of HD, in contrast, have not previously been reported. Such associations are of interest, however. Behavioural changes are highly complex, involving functions associated with a diversity of brain areas, so might be expected to be associated with extrastriatal pathology to a greater extent than motor features. Psychiatric disorders are among the aspects of HD most amenable to treatment, but predicting which patients will develop psychiatric dysfunction is challenging, and the identification of consistent structural correlates of such dysfunction could be of value in supporting clinical risk stratification.

A cross-sectional VBM analysis of volumetric MRI data was therefore carried out on baseline MRI scans from the longitudinal imaging study cohort. This analysis aimed to examine associations between regional grey matter volume and clinical disease measures, focusing on motor and behavioural dysfunction, using strict correction for multiple comparisons across the whole brain. Cross-sectional whole-brain VBM analyses of raw regional volume differences between the subject groups in this cohort have been published elsewhere (Henley *et al.* 2008) and are therefore not discussed here. In addition, the use of the novel Short Behavioural Assessment (SBA) in parallel with the Beck depression inventory (BDI) in all subjects in the study enables a preliminary analysis of the degree of concordance of the newer behavioural measures in comparison with the more established tool.

VII.2 Contributions and collaborations

Subject recruitment and characterisation and MRI scanning were as for the work presented in the previous chapter. Image processing was carried out by Ms Susie Henley. All other work, including data analysis and interpretation and preparation of figures was carried out by the author.

VII.3 Subjects and methods

VII.3.1 Subjects

The analysis included subjects from the baseline assessment of the longitudinal imaging study, whose MRI scans were of sufficient quality. This amounted to 81 subjects whose demographic characteristics were as shown in Table 15, page 175.

VII.3.2 Clinical assessments

Motor features were assessed using the UHDRS motor score (chapter II.5.1, page 79 and Appendix D). The behavioural measures analysed were the Beck depression inventory (Appendix F) and the Short Behavioural Assessment (Appendix G).

VII.3.3 MR imaging

1.5 Tesla T1 volumetric MRI scans were acquired and preprocessed as described in chapter II.7.1, page 81. Images were processed using MATLAB 7.0 (The MathWorks, Inc, Natick, Massachusetts, USA) and SPM2 (Wellcome Department of Cognitive Neurology, ION, London). VBM was performed following a modified version of the optimised method (Good *et al.* 2001).

VII.3.4 Analysis of clinical scores

Total scores for the SBA were calculated by summing the frequency and severity scores in each domain. Differences in clinical scores across groups were analysed using linear regression models as described in II.11.7, page 89, with age and gender as covariates. Initial comparison of the BDI and SBA assessments was performed by examining the correlation between them in a given subject; because the units of both scales are arbitrary, comparing the two measures against a line of unity is not meaningful, so a regression fit line was plotted. Since the strength of a correlation between two measures is not necessarily the best indicator of their ability to measure a given feature, a Bland-Altmann analysis (Bland *et al.* 1986) was also performed: for each subject, the difference between the two measures was plotted on the vertical axis against the mean of the measures on the horizontal axis. Values for the bias (mean difference between the measures) and limits of agreement (mean ± two standard deviations of the difference) were calculated.

VII.3.5 Image analysis

Initial processing used a modified version of the script provided by Christian Gaser (Gaser 2008). Native space images were affine-registered using the standard SPM2 T1 template, and underwent initial segmentation. Normalisation parameters were estimated for warping the resulting grey matter segments onto the SPM2 grey matter template. These normalisation parameters were then used to warp the original native space images. Normalised images were then segmented into grey matter, white matter and cerebrospinal fluid (CSF) and these segmentations were modulated using

the volume changes from the normalisation. White matter and CSF segments were not used in any further analysis as the primarily focus was on grey matter changes.

Preliminary work showed that normalising grey matter to a grey matter template resulted in better alignment of the grey matter segments compared with standard normalisation (judged visually and by looking at the 'reverse contrast', i.e. assessing where patients appear to have more grey matter than controls). This was achieved with the standard SPM templates; using customised (study-specific) templates for normalisation and segmentation did not produce any additional improvement. Each grey matter segment then had spurious non-brain tissue removed according to a brain mask derived from the corresponding original image using MIDAS semi-automatic segmentation software (Freeborough *et al.* 1997). Finally the images were smoothed using an 8mm full width half-maximum Gaussian kernel.

VII.3.5.1 Analysis of grey matter volume associations

A regression model, using age, gender and TIV as covariates, was used to examine associations between grey matter volume and the clinical variables of interest. The model used was of the form

$$Volume = \beta_1 x + \beta_2 age + \beta_3 gender + \beta_4 TIV + \mu + \varepsilon$$

where x is the clinical measure of interest, μ is a constant term and ε represents between-subject variability. The contrast of interest was $\beta_1<0$, i.e. regions where there was a significant negative association between grey matter volume and x.

An explicit mask was applied to SPMs to exclude any voxels for which more than 10% of the images had a value of less than 0.1. This was preferred to the default 'absolute' mask option in SPM, which would exclude any voxels for which one or more images had a value of less than 0.1 and thus perhaps be unduly influenced by a single poorly-registered scan. All SPMs were thresholded using a false discovery rate (FDR) of $q<0.05$ to correct for multiple comparisons and displayed as overlays on a smoothed version of the MNI-152 T1 template. In order to localise regions of atrophy, MNI coordinates were converted to Talairach space and localised to anatomical regions using a publicly available online atlas (International Neuroimaging Consortium 2008).

The associations examined were behavioural scores (BDI and SBA) across all gene carriers, and UHDRS motor score in the manifest HD group.

VII.4 Results

Demographic and clinical characteristics of subjects, and inter-group matching, were as shown in Table 15 (chapter VI.6.2, page 175).

UHDRS motor score (Figure 46A) was significantly higher in the early HD group than both controls and premanifest subjects (p<0.001 for both) but did not differ significantly between controls and premanifest subjects (p=0.16). Despite a trend to increase, there were no significant differences between groups in BDI score (Figure 46B: controls v. premanifest, p=0.48; controls v. early HD, p=0.14; premanifest v. early HD, p=0.55). Total SBA scores (Figure 46C) were significantly higher in early HD than in controls (p=0.041) but the premanifest HD (p=0.20) did not differ significantly from controls (p=0.57) or early HD (p=0.20).

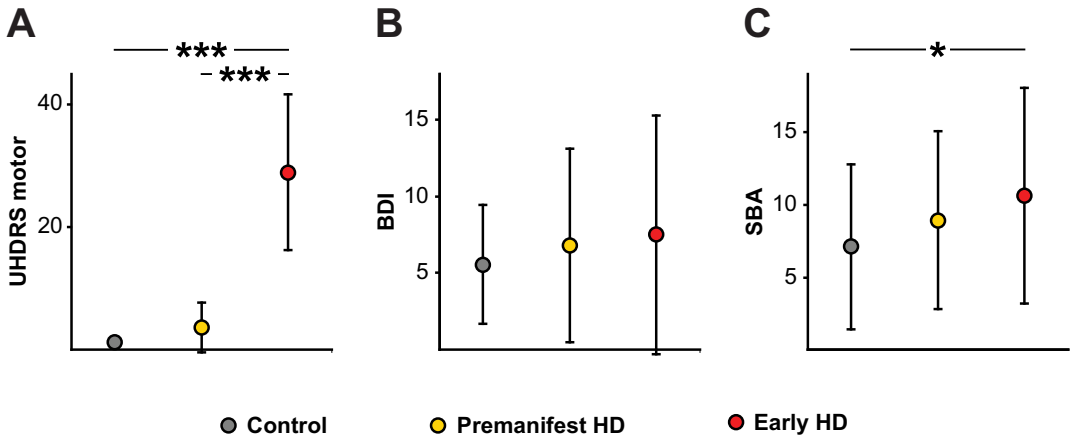

Figure 46 Clinical scores by group

Graphs show group means while error bars represent standard deviation. A. UHDRS motor score. B. Beck depression inventory score. C. Short behavioural assessment score. *p<*0.05,* ***p<*0.001 by linear regression.*

VII.4.1 Comparison of BDI and SBA

Comparisons of each subject's Beck depression inventory and short behavioural assessment score are given in Figure 47. The scatter plot (Figure 47A) reveals a moderate correlation (R=0.67); the

Bland-Altmann plot (Figure 47B) did not reveal an obvious relation between the difference and the mean, indicating a lack of systematic magnitude-related bias; the mean bias was +2.0 (95% CI 0.7 to 3.2) and the limits of agreement (mean ± two standard deviations) were -9.4 to 13.3.

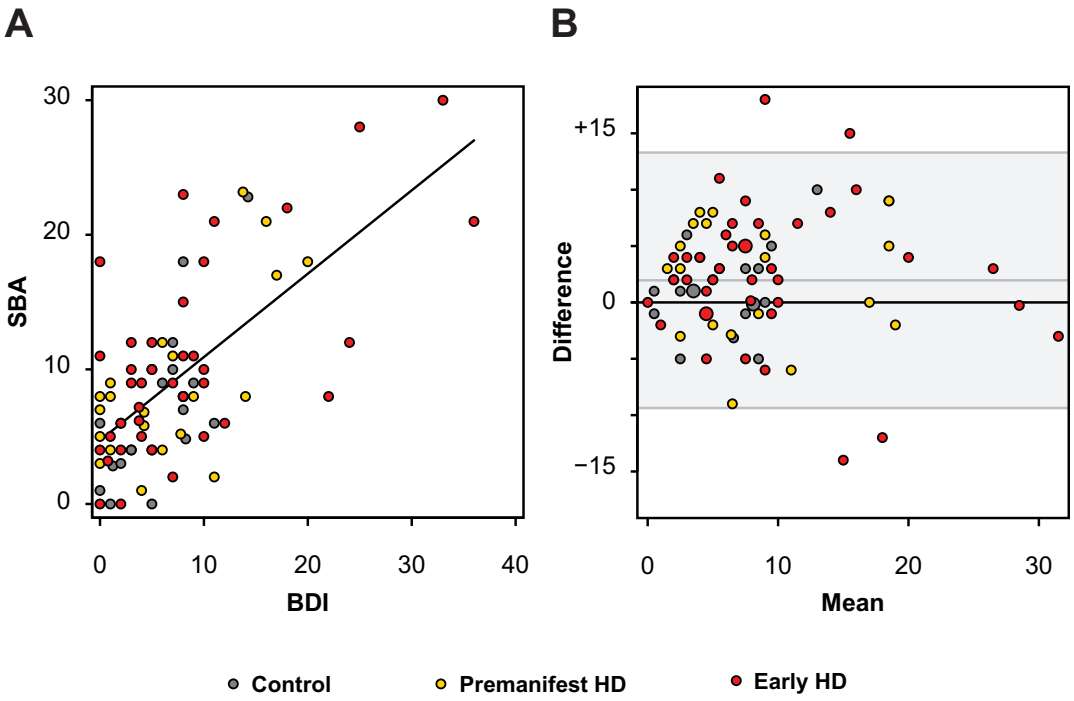

Figure 47 Analysis of the agreement between Beck depression inventory and Short behavioural assessment scores

***A**. Scatter plot of subjects' SBA scores against Beck scores, with linear regression best-fit line. **B**. Bland-Altmann plot, showing difference between the two scores (SBA - BDI) on the vertical axis against mean of the two scores on the horizontal axis. The grey region depicts the mean ± two standard deviations.*

VII.4.2 VBM analysis of clinical features

In manifest HD subjects, there were significant associations between grey-matter volume and UHDRS motor score in both caudate nuclei. The largest area of association was in the right precentral gyrus, corresponding to the primary motor cortex (Figure 48). VBM analysis revealed no brain regions where score on either of the behavioural scales in all HD gene carriers was associated with grey-matter volume after correction for multiple comparisons (data not shown).

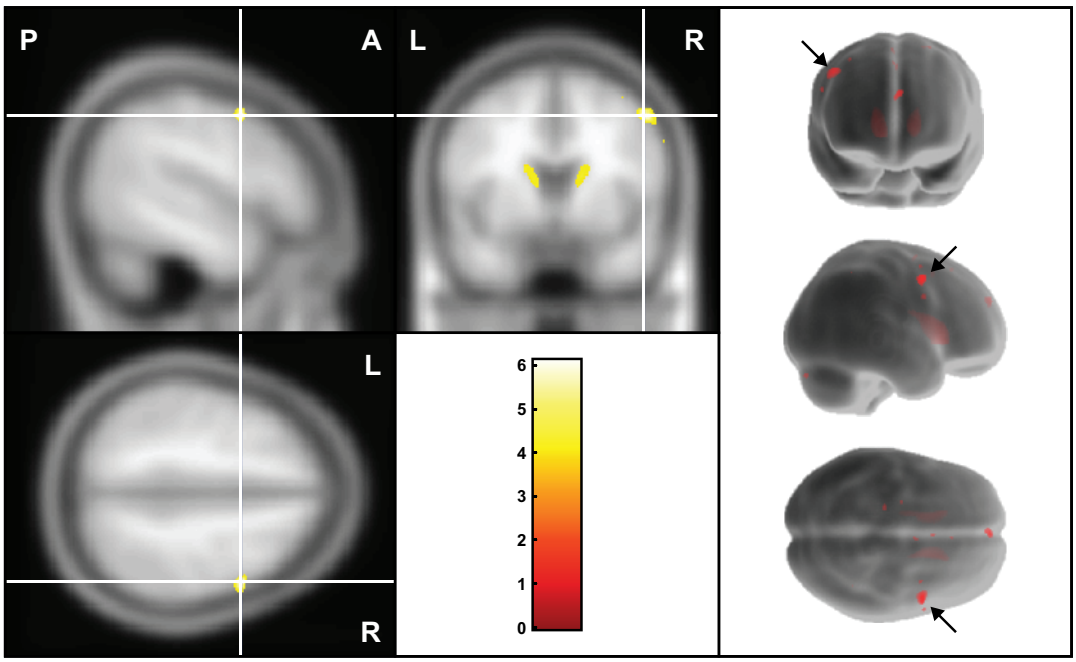

Figure 48 Statistical parametric maps (SPMs) showing regions where grey matter volume was negatively correlated with UHDRS motor score in early HD patients

Significant associations are seen in both caudate nuclei and the right primary motor cortex (crosshair and arrows). The colour bar shows t-score. The SPM is thresholded using false discovery rate (FDR) correction for multiple comparisons with q<0.05. A, anterior; P, posterior; L, left; R, right.

The finding that lower grey-matter volume in the right, but not left, primary motor cortex was associated with higher UHDRS motor score led to the development of a hypothesis that this asymmetry may be reflected in the clinical phenotype of manifest HD, the expectation being that left-sided motor signs may be more severe in a given patient.

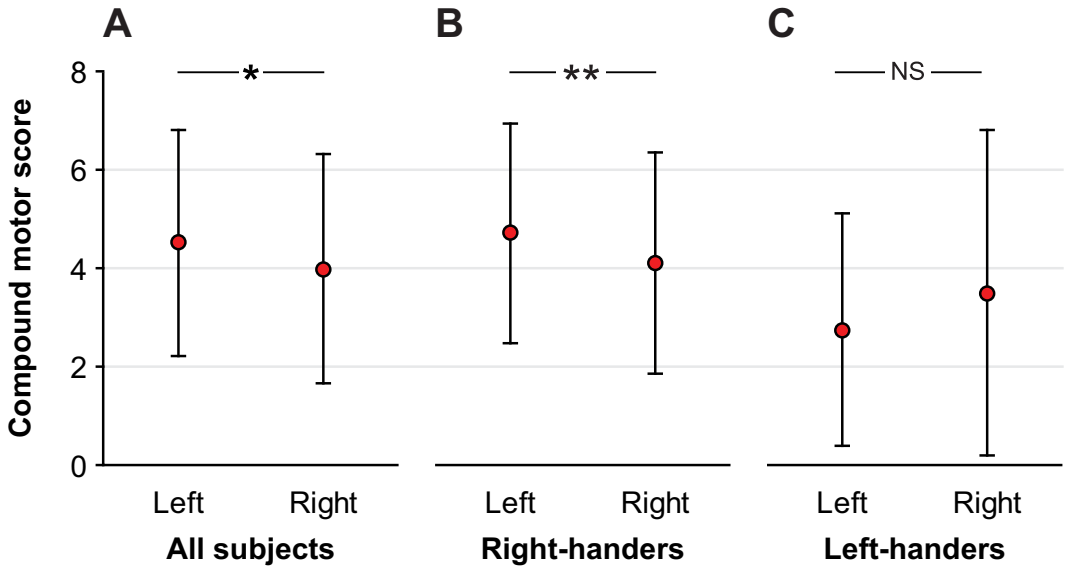

Figure 49 Post-hoc analysis of asymmetry of motor signs in the early HD group

***A**. All subjects. **B**. Right-handed subjects only. **C**. Left-handed subjects only. Graphs show mean compound upper limb motor score with standard deviation error bars. Two-tailed paired t-tests: NS, not significant; *p<0.05; **p<0.01.*

Accordingly, a post-hoc analysis was performed to test this hypothesis. Compound motor scores for the left and right upper limbs were constructed for each subject by adding the rigidity, pronate-supinate, finger-tapping, dystonia and chorea scores for each limb, such that the maximum score per limb was 20 points. A paired two-tailed t-test was then used to test whether, within each subject, there was a significant difference in motor severity between the upper limbs. Scores for the left upper limb were significantly higher than those for the right (Figure 49A and Table 23; p=0.01). When the analysis was restricted to right-handed subjects (Figure 49B), the difference was more pronounced (p=0.002). In the four left-handers (Figure 49C), the trend was reversed, though the left-right difference was not significant (p=0.22). In control subjects, there were no significant differences in motor score between left and right upper limbs in all subjects or right-handers only (p=0.33 for both).

Early HD patient subgroup	N	Compound upper limb motor score, mean ± SD		Paired t-test, *p*
		Left	Right	
All	40	4.53 ± 2.30	4.05 ± 2.33	0.01
Right-handers	36	4.72 ± 2.24	4.11 ± 2.25	0.002
Left-handers	4	2.75 ± 2.36	3.50 ± 3.32	0.22

Table 23 Post-hoc analysis of asymmetry of motor signs in the early HD group

To test whether this apparent effect of hand dominance, rather than left-right lateralisation *per se*, on asymmetry of motor signs was statistically significant, two scores were produced for each subject: a left-right difference score, calculated by subtracting the right from the left compound motor score; and a nondominant-dominant difference score, calculated by subtracting the compound motor score in the dominant limb from that in the nondominant limb. A paired t-test was then used to test whether these scores were significantly different, i.e. whether hand dominance has a greater effect on motor asymmetry than does pure left-right lateralisation. The mean difference was higher for the dominance score than the left-right score (0.63 ± 1.05 versus 0.48 ± 1.14 respectively) but the difference was not statistically significant (p=0.18). Comparing the difference scores between HD patients and controls, the patients displayed significantly more asymmetry than controls in the dominance score (p=0.038) but not the left-right score (p=0.16)

VII.5 Discussion

The analysis of cross-sectional motor abnormalities confirmed the expected findings in the three groups, showing a lack of significant difference in total UHDRS motor score between controls and premanifest HD, but with early HD subjects having significantly higher scores than the other two groups. This is in keeping with the definition of manifest HD and the findings of others in premanifest and early HD (Marder *et al.* 2000; Witjes-Ane *et al.* 2007).

The parallel administration of the BDI and SBA allows a preliminary analysis of the degree of concordance of the two measures. For both measures, as expected, there was a trend for scores to increase, indicating more severe behavioural disturbance, from control to premanifest to early HD. For BDI, there were no significant differences in score between any groups, but for the SBA score

the early HD group had significantly higher scores than controls. This is likely to indicate that the SBA is better able to detect the broad spectrum of behavioural features in HD, whereas the BDI focuses only on the affective domain, and also has the potential to be confounded by physical manifestations of HD. Of course, it is possible that the significant difference in SBA in early HD is erroneous, and the BDI is giving a truer measure of behavioural dysfunction but this does not seem likely given that the SBA is derived from the larger, well-validated and HD-specific Problem Behaviours Assessment (Craufurd *et al.* 2001).

Comparing the two measures directly is difficult for several reasons. Firstly, the scores on both scales are arbitrary and cannot be compared directly. Secondly, as alluded to above and in chapter II.11.9, in the absence of a 'gold standard', in a situation where the outcomes of an existing and a new measure are found to correlate, it is impossible to determine objectively whether any discrepancy between them means that the new measure is better or worse at evaluating the underlying disease: additional information about the nature of the tests and their ability to access known disease features is required. Moreover, as Bland and Altmann point out, examining the correlation between two such measures is unlikely to be informative alone unless the correlation is exceptionally poor, since any two measures of the same feature are likely to correlate well (Bland *et al.* 1986). The Bland-Altman plot (Figure 47) is a more informative means of comparing agreement between two tests, since it enables examination of the difference between the two scores over the range of (mean) scores, highlighting any tendency for systematic differences and showing the spread of differences more clearly than a scatter plot. Note that the absolute value of the difference is not meaningful for the comparison between BDI and SBA scores, since the values are arbitrary. Nonetheless it is encouraging that there is no obvious tendency for the difference between the scores to be influenced by their mean value.

Overall, it seems reasonable to conclude from these data that the SBA is capable of capturing a disease-related signal of behavioural abnormality in early HD that was not detected by the BDI and that it does not appear to introduce any systematic bias into behavioural assessment over the range of behavioural phenotypes in this cohort. These conclusions are of course highly preliminary, but further study of the intra-and inter-rater reliability of the SBA and its ability to detect behavioural disturbance that is clinically important is warranted.

The VBM analysis of behavioural score in HD gene carriers highlighted no areas where behavioural dysfunction on either scale was significantly associated with regional grey-matter volume. The regional neuroanatomical correlates of behavioural dysfunction in HD have not previously been studied. Some attention has focused on the somewhat overlapping field of cognitive dysfunction: for instance, in a complementary VBM study based on this same longitudinal imaging cohort, Henley and colleagues found that poorer performance on tests of facial emotion recognition was associated with reduced volume in the striata for all emotions and, in addition, defective fear recognition was associated with additional atrophy of the right insula and both lateral orbitofrontal cortices, implying that atrophy of both generic and localised regions may underlie the emotion recognition deficits in HD (Henley *et al.* 2008) In contrast to both specific neuropsychological functions and motor impairment, however, psychiatric and behavioural features are such complex and integrated phenomena that association with specific brain regions seems unlikely. Nevertheless, some authors have asserted that involvement of frontal-subcortical circuits is likely to be a main contributor to behavioural disturbance in HD (Bonelli *et al.* 2007), with some support from functional studies showing that deficits of functional connectivity show associations with measures of behavioural dysfunction in the basal ganglia (Wolf *et al.* 2008).

In this context, there are three possible interpretations for the lack of significant associations of regional atrophy. The first possible explanation is that there is a true, single regional basis to the behavioural dysfunction of HD that was missed by this analysis, perhaps through a lack of statistical power. The second is that different structural deficits may underlie different forms of behavioural dysfunction in HD (by analogy to the differential structural associates of facial fear recognition), but that the population under study, and their behavioural deficits, were too heterogeneous to allow a subgroup analysis of individual behavioural deficits. The final possibility is that the behavioural dysfunction is caused by deficits in neural networks too disseminated to produce specific regional volume changes detectable by such a whole-brain analysis. *A priori*, it is likely that each of these possible explanations contributes to the lack of regional signal in this analysis: subcortical-frontal networks, which are known to be dysfunctional in HD, probably do contribute a significant component to behavioural problems but, because of the heterogeneity of behavioural dysfunction, atrophy in these specific areas may not correlate closely with specific measures of behavioural performance; finally there may be more widespread cortical networks whose dysfunction (or

atrophy) may superimpose significant additional burdens on behaviour. Because of a lack of regional associations with either behavioural scale in total, it was not felt appropriate to examine for associations with different subscales. Overall, the negative outcome from this analysis suggests the identification of neuroanatomical substrates of behavioural dysfunction in HD is likely to require further study in much larger patient cohorts.

In the analysis of motor function, higher UHDRS motor scores were associated with lower grey matter volumes in both caudate nuclei and the right precentral gyrus. Bilateral caudate involvement has been a consistent finding from previous VBM studies of motor associations but the extrastriatal associations have been difficult to synthesise between studies, partly because of their significant methodological differences. The precentral gyrus has not previously been implicated in a VBM analysis of motor features of HD, and previous work has generally highlighted regions such as the hippocampi and hypothalamus, which have little direct connection with motor function, above areas with plausible motor roles such as the cerebellum (Kassubek *et al.* 2004; Jech *et al.* 2007). The related morphometric technique of cortical thickness measurement is perhaps a more statistically powerful way of examining cortical involvement and suggests that, with the exception of the temporal lobes, widespread cortical atrophy contributes to the motor phenotype in HD, without highlighting either precentral gyrus as being differentially affected. Neuropathological studies do little to clarify matters: the prefrontal cortex has consistently been shown to display more severe neuropathological abnormalities than the motor cortex (e.g. van Roon-Mom *et al.* 2006), though of course neuropathology and relative functional impairment need not be strongly associated. Nonetheless, the present VBM study used conservative analysis techniques with robust whole-brain compensation for multiple comparisons, so its findings may be considered to reflect genuine structural associations with motor impairment, after correction for the effects of age, and do suggest that disease-related atrophy of this critical motor region may contribute directly to the motor phenotype of HD. As the site of the primary motor cortex, with its critical role in the execution of voluntary movements, the finding of significant association here between atrophy and motor dysfunction is of interest. Moreover the existence of direct glutamatergic excitatory projections from the primary motor cortex to the striatum (Albin *et al.* 1989) raises the possibility that degeneration in the two regions identified by this VBM analysis may be linked, perhaps by glutamatergic

excitotoxicity which has been proposed as a major contributor to cell dysfunction and death in the striatum (Estrada Sanchez *et al.* 2008).

The asymmetry of the cortical findings in this analysis is of interest as well: atrophy of the left, but not the right precentral gyrus (governing movement of the right side of the body) was associated with motor impairment. While this study was not powered for a direct comparison of left versus right asymmetry in grey-matter volume, this would be an interesting analysis for future work.

Previous studies have identified asymmetries in neuroimaging abnormalities in HD. A recent morphometric study of the basal ganglia found significantly more atrophy on the left (Muhlau *et al.* 2007) and proposed that this was due to an increased lifetime's use of the dominant hemisphere leading to increased excitotoxicity; while an MR spectroscopy study revealed elevated lactate levels that were markedly higher on the left (Jenkins *et al.* 1998). In the present analysis, it was the right-sided cortex that displayed an association with motor score which is in contrast to the previous left-sided findings of others in the basal ganglia. While Muhlau and colleagues showed that that the degree of asymmetry in the basal ganglia correlated with the total UHDRS motor score, no study has attempted to investigate whether asymmetry of atrophy is matched by a corresponding asymmetry in motor function.

The *post-hoc* analysis of the hypothesis (based on the VBM results) that left-sided upper limb motor function would be more impaired than that on the right revealed that in the early HD group, left-sided compound motor scores were significantly worse than those on the right, when compared within each subject. This difference remained significant when confined to right-handed subjects only, and in left-handed subjects the trend was reversed but the difference was not significant, probably because there were only four such subjects. Nonetheless, this initial analysis confirms that the lateralisation of atrophy-motor associations changes was reflected by an asymmetry of the motor phenotype. It also raises the suggestion that the non-dominant limb, rather than the left limb *per se*, may be differentially affected in early HD, though this was not significant when the two differences were compared directly.

It could be argued that all functions will be less well-performed with the non-dominant limb, and that the difference in the HD group is not HD-related. However, in control subjects, there were no

significant asymmetries in motor score and when the difference in motor score between the dominant and nondominant limbs for each subject was compared between subject groups, the early HD patients displayed a significantly greater degree of asymmetry than did controls. Overall, therefore, based on the post-hoc asymmetry analysis of upper limb motor function in this cohort, it can be concluded that the VBM findings of left-sided primary motor cortex asymmetry across the cohort correspond to an underlying tendency for the nondominant limb to have significantly worse motor function in a given HD patient.

On the face of it, these findings may seem incompatible with the previous left-sided (i.e. dominant hemisphere) structural and MRS findings of others. However, one possible explanation is a dissociation between upper limb motor function and striatal pathology, such that motor cortical atrophy is leftward-biased, corresponding to worse right-sided limb dysfunction, while striatal pathology may be rightward-biased but does not cause limb motor asymmetry. This does not imply, of course, that the striatum does not contribute to motor dysfunction but that the motor cortex may be a greater contributor to limb asymmetry than is the striatum.

In addition, this VBM analysis and that of its motor correlates affirms the importance of extrastriatal atrophy in the clinical manifestations of HD, arguing in favour of further investigation of the regional correlates of clinical dysfunction as well as the clinical necessity for the inclusion of atrophy measures incorporating all brain regions in the repertoire of potential biomarkers for progression in HD.

VII.6 Publications relating to this chapter

The work presented here is under preparation for submission. VBM analyses of these baseline data for the cohort, focusing on genetic and psychological measures, have been published as:

- Henley SMD, Wild EJ *et al*. (2008) *Defective emotion recognition in early HD is neuropsychologically and anatomically generic*. **Neuropsychologia** 46(8): 2152-2160.

- Henley SMD, Wild EJ *et al*. (2009) *Relationship between CAG repeat length and brain volume in premanifest and early Huntington's disease*. **Journal of Neurology** In press.

Chapter VIII Conclusions and future work arising from this thesis

VIII.1 The need for biomarkers

IN DESCRIBING THE clinical features of Huntington's disease, the introductory chapter of this thesis set out the pressing need for treatments that will slow its progression, and indicated how such treatments, if offered judiciously to the premanifest gene carriers most likely to benefit from them, could ultimately lead to a cure for HD. The introduction went on to argue that because of the disease's relative rarity, slow progression, long premanifest phase and clinical heterogeneity, the shortcomings of our clinical assessment tools, and the large number of competing potential therapeutic approaches, a conventional approach to the establishment of disease-modifying therapies is likely to be inadequate, and biomarkers that may be able to enhance the speed, efficiency, economy and robustness of clinical trials will be needed. In the remainder of the chapter, the literature on imaging, biofluid and quantitative clinical biomarker candidates to date was reviewed, laying the foundation for the subsequent chapters.

VIII.2 Immune activation and plasma biomarkers

The work presented in chapters III and IV, taken together, describes the identification and elucidation of a novel pathogenic pathway in HD. This work began as a proteomic discovery project without specific *a priori* hypotheses beyond the proposal that there may be significant, disease-related differences in protein expression between HD and control plasma detectable by careful proteomic profiling, and the principle, set out in Chapter I, that a plausible mechanistic connection with the pathogenesis of a disease is a *sine qua non* for the establishment of biomarker candidates. The study identified eighteen proteins with significantly differential disease-related expression. Three of these candidate proteins were further studied for their ability to behave as biomarkers and the most promising, clusterin, was shown to behave in human plasma and CSF to track with disease severity in HD.

The specific proteins most easily identified by whole-proteome discovery techniques may not be those best suited for use as possible biomarkers, because of the incomplete coverage of the proteome that any such technique can offer. However, when viewed together, such findings can

offer an overall impression of the nature of the plasma protein profile of HD. Strikingly, almost all of the proteins identified as having altered expression were components of the innate immune system and acute phase response, raising the possibility of a widespread and progressive inflammatory derangement in HD. The study of this possibility formed the basis for the ensuing work, with a focus maintained on the possible utility of inflammatory changes as possible biomarkers. Using an ELISA technique, levels of IL-6 were shown to increase significantly with advancing HD and were shown to be elevated by 12 weeks in the R6/2 mouse. IL-6 is a key pro-inflammatory cytokine whose regulation is governed by the NFκB pathway, which has previously been implicated in the cellular pathobiology of HD (Khoshnan *et al.* 2004). The work presented in Chapter IV demonstrates that cytokines and chemokines are dysregulated in HD and that a direct effect of mutant huntingtin, acting within the myeloid cell, is sufficient to explain this immune activation, which was also seen in three different HD mouse models. The elevation of IL-6 levels in plasma from premanifest HD subjects suggests that it may be a very early event related to neuronal dysfunction in HD.

The exploration of inflammatory molecules such as cytokines as possible biomarkers must be treated as preliminary data only, and its findings interpreted with caution, as it is based on samples derived from a single population, studied cross-sectionally only. However, these initial findings do appear promising. Several cytokines and chemokines tested appear to track with disease stage and clinical severity. The early elevation of IL-6 levels suggests a possible use for IL-6 as a cross-sectional state biomarker of the kind represented in Figure 10A-B: a therapy aimed at reducing expression of mutant huntingtin or modulating the NFκB pathway could be monitored for intracellular effect by studying whether treatment was associated with a return of IL-6 levels towards those seen in the control population. Meanwhile, the stepwise logistic regression and ROC curve analyses presented suggest a means for identifying and comparing combinations of biomarkers, both within and between modalities, and suggests that doing so is likely to result in combinations with greater ability to detect clinically significant differences than single markers.

Three key aspects of this novel pathogenic pathway require further study: further characterisation of the pathway; inflammatory biomarkers; and its therapeutic potential.

First, though the present work demonstrates that mutant huntingtin causes hyperactivity of myeloid cells, and the work of Khoshnan and colleagues suggests that the NFκB pathway may be a possible

means by which such dysfunction is mediated, the exact link between the mutant protein and the functional overactivity of monocytes requires systematic investigation. A followup project is already underway to investigate whether the stimulation of monocytes from HD gene carriers results in increased nuclear entry of NFκB. Meanwhile, the IκB kinase / NFκB signalling pathway is not the only means by which IL-6 release is triggered in myeloid cells: the JAK/STAT pathway is a capable of producing independent cytokine production, but is also linked to the NFκB pathway via SOCS protein regulation. Work is underway using novel fluorescent flow cytometry techniques in HD monocytes to determine whether components of the JAK/STAT pathway are dysregulated in HD.

Second, before inflammatory molecules can be used as biomarkers, greater knowledge is required of how they behave in HD. Significant insight can be gained from full dissection of the pathogenic pathways involved, as described above. Specific studies to evaluate the feasibility of inflammatory markers will also be required. The present work deals only with cross-sectional changes, but longitudinal evaluation of biomarker candidates is required. The changes seen in cytokine levels are small — only one- or two-fold differences in level between clinical disease stages it would usually take an individual HD patient several years to progress to. Longitudinal study is required to establish the intra-subject variability and whether each subject's cytokine profile tends to increase linearly with progression and, if so, over what interval such changes may be detected and with what clinical changes they correspond. Work is underway to amass a suitably large collection of plasma samples obtained longitudinally from well-characterised subjects, and analyse them using the multiplex ELISA platform according to the principles set out in Chapter II.11.13. In addition, validation of the present cross-sectional findings from one or more independent populations is required, to ensure that the findings presented are not partially or wholly artifactual, due to such factors as genetics, diet, environmental exposure, or medications.

Both of these aims are best achieved in the context of large, multi-centre biomarker studies of the kind that are already in progress. One such study, TRACK-HD, has been designed with the evaluation of plasma biomarker candidates specifically in mind, and incorporates rigorously standardised plasma collection, processing and storage requirements to yield high quality and highly consistent plasma samples from four sites internationally, both cross-sectionally and longitudinally. Furthermore, the multimodal phenotypic assessment of subjects, with clinical, imaging and

quantitative motor evaluation, will greatly enhance our ability to evaluate inflammatory markers, both alone and in combination with other markers from both plasma and other assessment modalities.

Third, but perhaps most importantly, if excessive inflammation is present both centrally and peripherally in HD, and is due to myeloid cell overactivity in the presence of mutant huntingtin, it is likely overall to be more harmful than beneficial, and the possibility of therapeutic interventions to suppress such inflammation warrants further study. Pharmacological anti-inflammatory treatments are already widely available and licensed for use in humans. Such treatments could be tested relatively easy in mouse models of HD; however, they are generally rather non-specific inhibitors of the activity of immune cells, typically lymphocytes. An innovative approach would be to examine the effect of bone marrow transplantation in HD animals, to see whether the replacement of mHtt-expressing monocytes and microglia with wild-type cells can result in an immune response centrally and peripherally that is more conducive to neuronal survival. The permanence and transferability to humans of such an approach is highly appealing and preparations for such work are underway.

Finally, from Chapter V it is apparent that plasma levels of neurofilament heavy chain protein are not biomarkers for HD. However, the study of neurofilament protein in CSF and brain remains of interest.

VIII.3 Global and regional brain atrophy

Chapter VI described the use of volumetric MR imaging techniques to measure whole-brain atrophy rate, and assess acceleration of atrophy, over two years in premanifest and early HD. In premanifest subjects far from onset, whole-brain atrophy rates do not appear to be significantly elevated, but from the cohort studied it cannot be concluded whether this is the case closer to onset. Rates of atrophy in early HD are significantly elevated, with atrophy occurring around five times higher than in control subjects.

What are the implications of these findings for the potential utility of whole-brain atrophy as a biomarker? First, because of the *a priori* advantages of whole-brain imaging over caudate volumetry — namely its ability to capture all clinically relevant brain atrophy, and the higher reproducibility of quantifying change in the whole brain, with its well-defined anatomical boundary — the finding of a

statistically significant increase in any subject group suggests that it will be valuable to apply the technique to future therapeutic trials involving subjects in that group. Measurement of caudate atrophy alone may be more sensitive to change in premanifest subjects, but further study of whole-brain atrophy using the BBSI is warranted to determine whether the technique is capable of measuring significant change in those subjects closer to disease onset, and whether atrophy rate ultimately predicts motor onset or other clinical features.

Acceleration of atrophy, meanwhile, is seen in early HD and appears to be associated with proximity to onset in premanifest subjects. These preliminary findings are based only on a small subset of subjects with two one-year image registrations, and therefore require study in larger populations.

Chapter VII presented a cross-sectional VBM study of the volumetric MR scan data from the cohort presented in the preceding chapter, adding information about regional atrophy to the whole-brain atrophy findings. Significant change in UHDRS motor score was significantly associated with atrophy in both caudate nuclei and the right precentral gyrus, and a post hoc analysis of lateralisation of motor signs confirmed a tendency for left-sided signs to be worse, in keeping with the pattern of atrophy. In contrast, there were no regions where behavioural scores were associated with atrophy rate, likely because of a lack of statistical power, the more distributed neural basis for behavioural symptoms, and the heterogeneity of such features in HD. This work argues that regional, as well as whole-brain approaches to volumetric imaging are likely to be of value in future observational and interventional studies in HD. Further, it suggests that a region-of interest approach to the study of motor features in HD, and indeed therapies expected to alter the motor phenotype, would be wise to focus on the basal ganglia and non-dominant motor cortical areas. Finally, if a regional basis for behavioural change exists and is capable of detection, very large cohorts of subjects are likely to be needed to unveil it.

VIII.4 Future directions

The search for biomarkers for HD has gained momentum in the past few years as we prepare for future disease-modifying therapeutic trials, and the vast amount of progress that has been made since then in the molecular neurosciences, combined with recent advances in neuroimaging, 'omics' discovery technologies and quantitative motor assessment, has fuelled a recent flood of

promising biomarker candidates. The challenges now faced include eliminating less useful candidates as well as identifying further candidates.

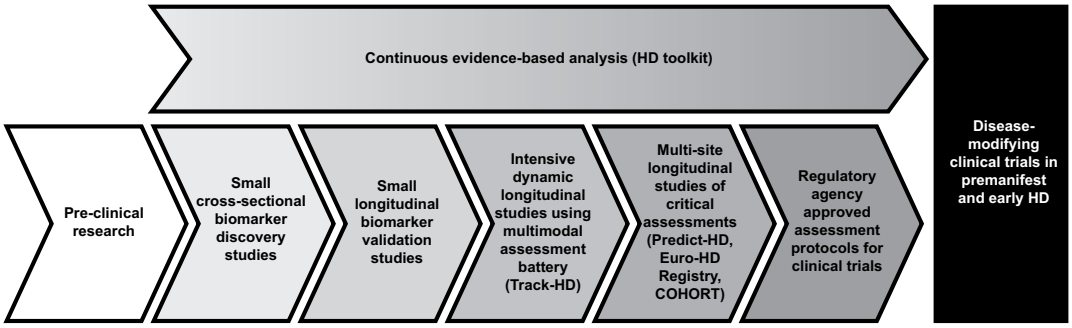

Figure 50 Pipeline for the establishment of biomarkers for HD as surrogate endpoints for clinical trials of disease-modifying therapies

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The pipeline presented in Figure 50 highlights the series of steps required for a potential marker to become an approved surrogate endpoint in a clinical trial of a putative disease-modifying therapy. Most of the findings discussed in the previous section are small cross-sectional or longitudinal studies. To date, few studies have evaluated multiple biomarker candidates under the same conditions, particularly in a longitudinal design. Differences in study design such as followup interval, sample preparation techniques, scan parameters, acquisition of clinical data and techniques for analysing and presenting data between studies make direct comparison of the relative ability of each marker to measure and predict the disease process impossible. Head-to-head comparison of candidate markers within and between modalities is now essential.

Unlike much of the work that has been performed to date, such direct comparisons require large sample sizes necessitating multi-centre projects, carefully standardised study design and monitoring, and multivariate statistical analysis. The infrastructure for such studies is already in place and several large longitudinal studies are ongoing. Each of these studies employs a large number of sites each carrying out a core of standardised assessments. The TRACK-HD study aims to act as an interface between small-scale biomarker discovery projects and these large-scale longitudinal trials, by comparing a large number of novel measures head-to-head in a few closely regulated centres. The most promising markers will then be carried forward into the more widely distributed longitudinal studies (TRACK-HD Steering Committee 2006-8).

It is to be anticipated that the first biomarker-powered multi-centre trials of disease-modifying treatments expected to delay onset in HD will begin within the next decade. The impressive results of candidate biomarker discovery to date must now be matched by rigorous mechanistic evaluation and systematic head-to-head comparison of biomarkers to maximise readiness for these trials. The author hopes that the work presented in this thesis may contribute in some small way to this pathway, as the global community of HD researchers inches towards the identification and adoption of successful disease-slowing treatments and towards the ultimate aim of all HD research: the cure.

VIII.5 Publications related to this chapter

Sections of this chapter are adapted from the following publications:

- Wild EJ and Tabrizi SJ (2008) *Biomarkers for Huntington's disease*. **Expert Opinion on Medical Diagnostics** 2(1): 47-62. Figure 50 is reproduced from this article by permission of Informa, Inc.

- Wild E, Björkqvist M and Tabrizi SJ (2008) *Immune markers for Huntington's disease?* **Expert Review of Neurotherapeutics** 8(12): 1779-1781.

Chapter IX Publications arising from this thesis

- Wild EJ and Tabrizi SJ (2006) *Predict-HD and the future of therapeutic trials*. **Lancet Neurology** 5(9): 724-5.

- Wild EJ, Petzold A, *et al*. (2007) *Plasma neurofilament heavy chain levels in Huntington's disease*. **Neuroscience Letters** 417(3): 231-3.

- Runne H, Kuhn A, Wild EJ, *et al*. (2007) *Analysis of potential transcriptomic biomarkers for Huntington's disease in peripheral blood*. **Proceedings of the National Academy of Sciences** 104(36): 14424-9.

- Dalrymple A, Wild EJ, *et al*. (2007) Proteomic profiling of plasma in Huntington's disease reveals neuroinflammatory activation and biomarker candidates. **Journal of Proteome Research** 6(7): 2833-40.

- Wild EJ and Tabrizi SJ (2007) *The differential diagnosis of chorea*. **Practical Neurology** 7(6): 360-73.

- Wild EJ and Tabrizi SJ (2008) *Biomarkers for Huntington's disease*. **Expert Opinion on Medical Diagnostics** 2(1): 47-62.

- Björkqvist M, Wild EJ, *et al*. (2008) *A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease*. **Journal of Experimental Medicine** 205(8): 1869-77.

- Arnulf I, Nielsen J, *et al*. (2008) Rapid Eye Movement Sleep Disturbances in Huntington Disease. **Archives of Neurology** 65(4): 482-8.

- Henley SMD, Wild EJ, *et al*. (2008) Defective emotion recognition in early HD is neuropsychologically and anatomically generic. **Neuropsychologia** 46(8): 2152-60.

- Leoni V, Mariotti C, *et al*. (2008) *Plasma 24S-hydroxycholesterol and caudate MRI in pre-manifest and early Huntington's disease*. **Brain** 131(11): 2851-9.

- Henley SMD, Wild EJ, *et al*. (2009) *Relationship between CAG repeat length and brain volume in premanifest and early Huntington's disease*. **Journal of Neurology**, published online ahead of print.

- Wild E, Bjorkqvist M and Tabrizi SJ (2008) *Immune markers for Huntington's disease?* **Expert Review of Neurotherapeutics** 8(12): 1779-81.

- Henley SMD / Wild EJ, *et al*. (2009) *Whole-brain atrophy as a measure of progression in premanifest and early Huntington's disease*. **Movement Disorders,** published online ahead of print.

- Wild EJ and Fox NC (2009) *Serial volumetric MRI in Parkinsonian disorders*. **Movement Disorders,** accepted for publication.

Appendices

Appendix A Demographic questionnaire

1. Status (Control, premanifest, HD)

2. Control status (partner/spouse, non-gene carrier)

3. Date of birth

4. Gender

5. Ethnicity:

- Asian or Asian British
  - Bangladeshi
  - Indian
  - Pakistani
  - Other
- Black or Black British
  - Black African
  - Black Caribbean
  - Black other
- Chinese
- White
  - White British
  - White Irish

    - White other
- Mixed
    - White/Asian
    - White/black African
    - White/black Caribbean
    - Other
- Other

6. Handedness (left, right, mixed)
7. Education level (None, CSE, O-level/GCSE, A-level, Technical diploma, University Entrance Diploma, College, Professional school degree, University degree, Higher degree, Other degree)
8. Years in education
9. Occupation
10. Occupation grade (Higher managerial/professional, Lower managerial/professional, Intermediate, Small employers, Self-employed, Lower supervisory/technical, Semi-routine, Routine, Never worked, Long-term unemployed, Medically retired)
11. Marital status (Single, partnership, married, divorced, widowed)

Appendix B Medical history questionnaire

1. Height (m)
2. Weight (kg)
3. History of birth or neonatal illness
4. History of childhood illness (<12 years)
5. History of adolescent illness (13-17 years)
6. History of adult illness
7. Major surgery
8. Units of alcohol per week
9. History of alcohol abuse (>21 units/week for males, >14 units for females: current, previous, never)
10. History of recreational drug use (current, previous, never)
11. Tobacco use (current, previous, never)
12. Cigarettes per day equivalent
13. Years of smoking
14. Date gave up smoking
15. Allergies
16. Current medications (name, dose, indication, duration)
17. Current comorbid conditions

Appendix C Huntington's disease history questionnaire

1. Affected parent (mother, father, both, unknown)
2. Estimated parental age of motor onset
3. Affected parent's current age or age at death
4. Patient's age at onset according to patient
5. Patient's age at onset according to family members
6. Patient's age at onset according to rater
7. Age at genetic test
8. First manifestation according to patient (None, Motor, Cognitive, Psychiatric, Oculomotor, Other, Mixed)
9. First manifestation according to family members (None, Motor, Cognitive, Psychiatric, Oculomotor, Other, Mixed)
10. First manifestation according to rater (None, Motor, Cognitive, Psychiatric, Oculomotor, Other, Mixed)
11. Small CAG repeat length
12. Large CAG repeat length
13. Analysing laboratory

Appendix D UHDRS motor scale

Motor scale

Item	Instruction	Score	Subsections
Gait	Observe the participant walking approximately 9 meters (10 yards) as briskly as they can, then turning and returning to the starting point.	0 normal gait, narrow base 1 wide base and / or slow 2 wide base and walks with difficulty 3 walks only with assistance 4 cannot attempt	Single score
Tandem gait	The participant is requested to walk ten steps in a straight line with the foot placed (accurately but not quickly) such that the heel touches the toe of the other foot. Deviations from a straight line are counted.	0 normal for 10 steps 1 1 to 3 deviations from straight line 2 More than 3 deviations 3 cannot complete 4 cannot attempt	Single score
Retropulsion test	The participant's response to a sudden posterior displacement produced by a pull on the shoulder while the participant is standing with eyes open and feet slightly apart is assessed. The shoulder pull test must be done with a quick firm tug after warning the subject. The participant should be relaxed with feet apart and should not be leaning forward. If the examiner feels pressure against his/her hands when placed on the participant's shoulders, the examiner should instruct the participant to stand up straight and not lean forward. The examiner should instruct the participant to take a step backward to avoid falling. Examiners must catch subjects who begin to fall.	0 normal 1 recovers spontaneously 2 would fall if not caught 3 tends to fall spontaneously 4 cannot stand	Single score

Item	Instruction	Score		Subsections
Tongue protrusion	Ask participant to open their mouth wide while you inspect it using a torch. Then ask participant to protrude their tongue well beyond their front teeth while keeping their mouth wide open and to keep it out as long as it takes you (as the examiner) to count aloud from 1 to 10. Participants should be made aware that they are not allowed to prevent their tongue from slipping back into the mouth by biting on it.	0	can hold tongue fully protruded for 10 sec	Single score
		1	cannot keep fully protruded for 10 sec	
		2	cannot keep fully protruded for 5 sec	
		3	cannot fully protrude tongue	
		4	cannot protrude tongue beyond lips	
Ocular pursuit	Should be assessed over a range of approximately 20° with a slowly moving target taking about 2 seconds to move from one shoulder to the other.	0	complete (normal)	Horizontal and vertical
		1	jerky movement	
		2	interrupted pursuits / full range	
		3	incomplete range	
		4	cannot pursue	
Saccade initiation	Should be tested over a 20° range, as for ocular pursuits. Saccade movement should be elicited by a sound (snapping fingers) or movement (wiggle fingers), but not by a verbal command to look to the right or left. If any head movements are made, subject should be prompted to keep head still.	0	normal	Horizontal and vertical
		1	increased latency only	
		2	suppressible blinks or head movements to initiate	
		3	unsuppressible head movements	
Saccade velocity	Should be tested at a larger range of approximately 30° so as to be able to detect incomplete range.	0	normal	Horizontal and vertical
		1	mild slowing	
		2	moderate slowing	
		3	severely slow, full range	
		4	incomplete range	

Item	Instruction	Score		Subsections
Rigidity	Rigidity is judged on passive movement of the arms with the participant relaxed in the sitting position.	0	absent	L and R
		1	slight or present only with activation	
		2	mild to moderate	
		3	severe, full range of motion	
		4	severe with limited range	
Finger taps	Participant taps thumb with index finger in rapid succession with widest amplitude possible, each hand separately. Count the full-size taps made over 5 seconds.	0	normal (≥15 in 5 sec.)	L and R
		1	mild slowing, reduction in amplitude (11-14 in 5 sec.)	
		2	moderately impaired (7-10 in 5 sec.)	
		3	severely impaired (3-6 in 5 sec.)	
		4	can barely perform task (0-2 in 5 sec.)	
Pronate/ supinate	Requires the participant to alternately hit the palmar and dorsal surface of one hand against the palm of the opposite hand. Use the palm of the opposite hand as a target. The participant should do this task as quickly as possible over a five-second interval. The task is graded according to the degree of slowing and irregularity.	0	normal	L and R
		1	mild slowing and / or irregular	
		2	moderate slowing and irregular	
		3	severe slowing and irregular	
		4	cannot perform	

Item	Instruction	Score		Subsections
Luria	Fist-hand-palm sequencing - Say 'Can you do this?' Examiner puts hand into fist on flat surface and sequences as follows: fist, side, flat (do not repeat this out loud). Watch to make sure that participant can mimic each step. When participant is able to join you then say 'Very good, now keep going, I am going to stop. Rest hand and start timing participant's sequences. A sequence is considered correct only if it is unaided by examiner and in the correct order. If participant is unable to complete any sequences over a 10-second period, then continue as follows. Say 'Now lets try it again. Put your hands like this. FIST; SIDE; FLAT'. Watch to make sure the participant can mimic each step. Using the verbal labels, begin the sequences again and ask the participant to 'Do as I do, Fist, Side, Flat' (repeat this as you continue). Continue to perform Luria 3-step. When participant is able to join you say 'Very good, now keep going, I am going to stop'. Rest hand and start timing participant's sequences. A sequence is considered correct if it is unaided by examiner model and in the correct order. Count completed sequences and score as above.	0 1 2 3 4	≥4 in 10 sec, no cue <4 in 10 sec, no cue ≥4 in 10 sec with cues <4 in 10 sec with cues cannot perform	Dominant hand only
Bradykinesia	Observe the participant during spontaneous motion such as walking, sitting down, arising from a chair, and executing the tasks required during the examination. This rating reflects the examiner's overall impression of bradykinesia.	0 1 2 3 4	normal minimally slow (?normal) mildly but clearly slow moderately slow, some hesitation markedly slow, long delays in initiation	Single score
Maximal dystonia	Maximal dystonia is defined here as a tendency toward a posture, posturing along an axis. Observe the participant during the examination; i.e., no particular manoeuvres are required to elicit these features. Maximal dystonia are typically observed during demanding motor tasks such as tandem gait. When rating dystonia facial dystonia (blepharospasm, jaw opening and closing) should be included in your assessment of the truncal region.	0 1 2 3 4	absent slight / intermittent mild / common or moderate / intermittent moderate / common marked / prolonged	Trunk, R upper limb, L upper limb, R lower limb, L lower limb

Item	Instruction	Score	Subsections
Maximal chorea	Maximal chorea is defined here as movement, not posture. Observe the participant during the examination; i.e., no particular manoeuvres are required to elicit these features. Maximal chorea is typically observed during demanding motor tasks such as tandem gait.	0 absent 1 slight / intermittent 2 mild / common or moderate / intermittent 3 moderate / common 4 marked / prolonged	Face, Buccal-oral-lingual, Trunk, R upper limb, L upper limb, R lower limb, L lower limb
Dysarthria	Observe speech throughout encounter	0 normal 1 unclear, no need to repeat 2 must repeat to be understood 3 mostly incomprehensible 4 anarthria	Single score

Diagnostic confidence score

Description	Score
Normal (no abnormalities)	0
Non-specific motor abnormalities (less than 50 % confidence)	1
Motor abnormalities that may be signs of HD (50 - 89 % confidence)	2
Motor abnormalities that are likely signs of HD (90 - 98 % confidence)	3
Motor abnormalities that are unequivocal signs of HD (≥ 99 % confidence)	4

(The Huntington Study Group 1996; Euro-HD Network REGISTRY Steering Committee 2003-8)

Appendix E UHDRS functional scales

(The Huntington Study Group 1996; Euro-HD Network REGISTRY Steering Committee 2003-8)

Total functional capacity (TFC)

Occupation

0 = unable, 1 = marginal work only, 2 = reduced capacity for usual job, 3 = normal

The participant's capacity to engage satisfactorily in gainful or voluntary works is assessed regardless of whether or not the participant is actually working. Normal refers to gainful employment, actual or potential, with usual work expectations. Reduced capacity refers to full or part-time gainful employment with lower than usual work expectations (relative to the participant's training and education), but with satisfactory performance. Marginal refers to a capacity only for part-time employment, actual or potential with low work expectations. Unable refers to a participant who would be unable to carry out these tasks, even with considerable assistance and supervision.

Finances

0 = unable, 1 = major assistance, 2 = slight assistance, 3 = normal

Assessed by surveying the participant's involvement in personal and family finances including balancing a chequebook, paying bills, budgeting, shopping, etc. Normal capacity refers to satisfactory handling of these basic financial tasks. Requires slight assistance refers to mild difficulties which would require the assistance / supervision of a family member or financial advisor. Requires major assistance refers to a participant who would require extensive supervision in handling routine financial tasks. Unable refers to a participant who would be unable to carry out these financial tasks, even with considerable assistance and supervision.

Domestic chores

0 = unable, 1 = impaired, 2 = normal

Refers to the participant's capacity to carry out routine domestic tasks such as cleaning, laundry, dishwashing, table-setting, cooking, lawn care, answering mail, maintaining a calendar, etc. Normal capacity refers to a full capacity without assistance. Impaired refers to impaired capacity requiring only slight assistance or supervision. Unable refers to marked incapacity requiring major assistance.

Activities of daily living

0 = total care, 1 = gross tasks only, 2 = minimal impairment, 3 = normal

Refers to the traditional areas of 'activities of daily living' (ADL) including eating, dressing and bathing. Normal refers to full capacity. Minimal impairment refers to impaired capacity requiring only slight assistance. Gross tasks only refers to impaired capacity requiring moderate assistance and supervision. Total care refers to major incapacity requiring total assistance and supervision.

Care level

0 = full time skilled nursing, 1 = home or chronic care, 2 = home

Refers to the most appropriate care environment to meet the participant's capacity, whether at home, at home or chronic care facility or full skilled nursing care (24 hours a day supervision).

Functional Assessment

Could the subject (one point each):

1. engage in gainful employment in accustomed work?
2. engage in any kind of gainful employment?
3. engage in any kind of volunteer or non-gainful work?
4. manage finances without help?
5. shop for groceries without help?
6. handle money as a purchaser in a simple cash transaction?
7. supervise children without help?
8. operate an automobile safely and independently?
9. do own housework without help?
10. do own laundry without help?

11. prepare own meals without help?

12. use the telephone without help?

13. take own medications without help?

14. feed self without help?

15. dress self without help?

16. bathe self without help?

17. use public transport without help?

18. walk to places in neighbourhood without help?

19. walk without falling?

20. walk without help?

21. comb hair without help?

22. transfer between chairs without help?

23. get in and out of bed without help?

24. use toilet / commode without help?

25. be cared for at home?

Independence Scale (IS)

100% No special care needed

90% No physical care needed if difficult tasks are avoided

80% Pre-disease level of employment changed or ended; cannot perform household chores to pre-disease level; may need help with finances

70% Self-care maintained for bathing; limited household duties (cooking, knives); cannot drive; unable to manage finances

60% Needs minor assistance in dressing, toileting, bathing; food must be cut

50% 24 hour supervision appropriate; assistance required for bathing, eating and toileting

40% Chronic care facility needed; limited self feeding, liquefied diet

30% Minimal input into own feeding, bathing, toileting

20% No speech, must be fed

10% Tube-fed, total bed care

Appendix F Beck depression inventory

(Beck *et al.* 1996)

Instructions
Read each item carefully, and tick the number next to the answer that best describes how you have been feeling **the past week including today**.
Please tick only **one answer** in each group.

A	I do not feel sad.	0
	I feel sad.	1
	I am sad all the time and can't snap out of it.	2
	I am so sad or unhappy that I can't stand it.	3

B	I am not particularly discouraged about the future.	0
	I feel discouraged about the future.	1
	I feel I have nothing to look forward to.	2
	I feel that the future is hopeless and that things cannot improve.	3

C	I do not feel like a failure.	0
	I feel I have failed more than the average person.	1
	As I look back on my life, all I can see is a lot of failure.	2
	I feel I am a complete failure as a person.	3

D	I get as much satisfaction out of things as I used to.	0
	I don't enjoy things the way I used to.	1
	I don't get real satisfaction out of anything anymore.	2
	I am dissatisfied or bored with everything.	3

E	I don't feel particularly guilty.	0
	I feel guilty a good part of the time.	1
	I feel quite guilty most of the time.	2
	I feel guilty all of the time.	3

F	I don't feel I am being punished.	0
	I feel I may be punished.	1
	I expect to be punished.	2
	I feel I am being punished.	3

G	I don't feel disappointed in myself.	0
	I am disappointed in myself.	1
	I am disgusted with myself.	2
	I hate myself.	3

H	I don't feel I am worse than anybody else.	0
	I am critical of myself for my weaknesses or mistakes.	1
	I blame myself all the time for my faults.	2
	I blame myself for everything bad that happens.	3

I	I don't have any thoughts of killing myself.	0
	I have thoughts of killing myself, but I would not carry them out.	1
	I would like to kill myself.	2
	I would kill myself if I had the chance.	3

J	I don't cry any more than usual.	0
	I cry more now than I used to.	1
	I cry all the time now.	2
	I used to be able to cry, but now I can't even cry even though I want to.	3

K	I am no more irritated by things than I ever am.	0
	I am slightly more irritated now than usual.	1
	I am quite annoyed or irritated a good deal of the time.	2
	I feel irritated all the time now.	3

L	I have not lost interest in other people.	0
	I am less interested in other people than I used to be.	1
	I have lost most of my interest in other people.	2
	I have lost all of my interest in other people.	3

M	I make decisions about as well as I ever could.	0
	I put off making decisions more than I used to.	1
	I have greater difficulty in making decisions than before.	2
	I can't make decisions at all anymore.	3

N	I don't feel that I look any worse than I used to.	0
	I am worried that I am looking old or unattractive.	1
	I feel there are permanent changes in my appearance that make me look unattractive.	2
	I believe that I look ugly.	3

O	I can work about as well as before.	0
	It takes an extra effort to get started at doing something.	1
	I have to push myself very hard to do anything.	2
	I can't do any work at all.	3

P	I can sleep as well as usual.	0
	I don't sleep as well as I used to.	1
	I wake up 1-2 hours earlier than usual and find it hard to get back to sleep.	2
	I wake up several hours earlier than I used to and cannot get back to sleep.	3

Q	I don't get tired more than usual.	0
	I get tired more easily than I used to.	1
	I get tired from doing almost anything.	2
	I am too tired to do anything.	3

R	My appetite is no worse than usual.	0
	My appetite is not as good as it used to be.	1
	My appetite is much worse now.	2
	I have no appetite at all anymore.	3

S	I haven't lost much weight, if any, lately.	0
	I have lost more than five pounds.	1
	I have lost more than ten pounds.	2
	I have lost more than fifteen pounds.	3

T	I am no more worried about my health than usual.	0
	I am worried about physical problems like aches, pains, upset stomach or constipation.	1
	I am very worried about physical problems and it's hard to think of much else.	2
	I am so worried about my physical problems that I cannot think about anything else.	3

U	I have not noticed any recent change in my interest in sex.	0
	I am less interested in sex than I used to be.	1
	I am much less interested in sex now.	2
	I have lost interest in sex completely.	3

Appendix G Short behavioural assessment

(After Thompson *et al.*)

Orientation (time)

Time of day morning / afternoon / evening; Day of week; Date; Month; Year

0 Fully oriented: knows time of day, day of week, date (within 3 days), month, year.

1 Partially disoriented

2 Disoriented: completely mistaken about time and day / date

3 Unable to respond because mute or cognitive deterioration too advanced

Orientation (place)

Name of venue; Approximate address of venue; Type of establishment

0 Fully oriented: knows name, approximate address, type of establishment

1 Partially disoriented

2 Disoriented: cannot correctly identify type of establishment

Orientation (person)

Identify 2 people

0 Fully oriented: identifies people known by name, and function of others; forgetting names acceptable unless known very well

1 Partially disoriented

2 Disoriented: wrongly identifies functions of people in the room

Depression

In the past month have you been feeling sad? (or down or blue?) Has your mood affected your daily activities? Have you found yourself doing something you would ordinarily enjoy and realised you are not having fun? Behavioural persuasion: sad voice or expression, tearfulness Start the interview with an open ended question. Additional questions re depressive syndrome: appetite, change in weight; difficulty or disturbance in sleeping? Does the depressed mood come and go or does it seem always to be there? Is there any change throughout the day?

Severity

0 Absent

1 Questionable or trivial

2 Low mood present intermittently but does not interfere with everyday function; rate 2 if subject can easily enjoy amusing activities or visits from friends

3 Feels sad much of the time, takes no pleasure from things that he / she usually enjoys, still able to cheer up sometimes with a big effort; low mood has definite effect on lifestyle, e.g. unable to enjoy company of friends or amusing diversions

4 Subject feels utterly miserable all day

Frequency

0 Never, or almost never

1 Seldom: <1 times a week

2 Sometimes: 1-4 times a week

3 Frequently: 5-6 times a week

4 Every day or almost every day

Anxiety

In the past month have you found yourself getting worried about things? Evidence of anxiety includes worrying, panic, feeling frightened or fearful for no apparent reason. Have you worried a great deal since your last visit? What is it like when you worry? Have you often felt on edge, or keyed up, or mentally strained? Have you had difficulty in relaxing? Do your muscles feel tensed up? When people get anxious they often feel their heart beating fast or they start shaking or sweating or can't get their breath. Have you had feelings like that?

Severity

0 Never

1 Questionable, vague unease (also rate 1 if subject's only worry or anxiety is about prognosis of HD)

2 Intermittent worry or anxiety, not severe enough to cause significant distress or interfere with everyday activities; rate 2 for mild anticipatory anxiety prior to social events or unfamiliar activities

3 Unpleasant anxiety present much of the time, significant impact on behaviour (e.g. avoids places or events associated with provoking anxiety)

4 Anxiety present all the time, major impact on lifestyle (e.g. agoraphobia, cannot leave home without an escort); rate 4 if regular panic attacks

Frequency

0 Never, or almost never

1 Seldom: <1 times a week

2 Sometimes: 1-4 times a week

3 Frequently: 5-6 times a week

4 Every day or almost every day

Suicidal thoughts

In the last month have you found yourself thinking that life is not worth living or that you would be better off dead? Have you thought about hurting yourself or killing yourself? Are you planning to hurt yourself or kill yourself? Have you taken any steps towards carrying out your plan? In the past four weeks, have you felt that life was not worth living or that you wouldn't care if you didn't wake in the morning? Have you thought about harming yourself or even making an attempt at suicide?

Severity

0 Absent

1 Questionable; also rate 1 if subject plans suicide at a later date when disease is more severe but obtains comfort from this as means to retain control of destiny

2 Sometimes very pessimistic with fleeting suicidal ideation

3 Pervasive and distressing feelings of hopelessness and more prolonged or frequent suicidal ideation, but has not yet acted on this in any way

4 Subject has attempted suicide or has made preparations such as saving up tablets or planning ways to avoid discovery when doing it

Frequency

0 Never, or almost never

1 Seldom: <1 times a week

2 Sometimes: 1-4 times a week

3 Frequently: 5-6 times a week

4 Every day or almost every day

Disruptive / aggressive behaviour

In the last month have you had any emotional or temper outbursts? Have you had times when you lost control of yourself? Have you hit, shoved or thrown things or expressed your temper in a physical way? Have you used threats or hostile words?

Severity

0 Normal

1 Questionable or trivial; within normal limits but worse than he / she used to be

2 Verbal outbursts outside socially acceptable limits, not causing significant problems / distress for other household members; becomes angry with self or inanimate objects when confronted with frustrating situations due to disability; violence towards property

3 Tantrums causing significant distress for household members or practical difficulties caring for subject; verbal hostility directed towards another person; explicit verbal threats of violence or behaviour causing a justifiable fear of personal violence

4 Temper tantrums so severe that relationship with carers is compromised, creating risk that subject will be rejected; any kind of actual physical assault

Frequency

0 Never, or almost never

1 Seldom: <1 times a week

2 Sometimes: 1-4 times a week

3 Frequently: 5-6 times a week

4 Every day or almost every day

Perseveration

Within the last month have you found yourself getting stuck on certain ideas? Within the past month have you been bothered by thoughts, images or fears that keep coming back even if you try not to have them?

Severity

0 Symptom absent

1 Questionable or trivial

2 Mild perseverative behaviours / abnormal preoccupations present; do not interfere with everyday life or cause significant distress to subject / carers; comes out with comments referring to earlier topic of conversation; rater observes perseverative phenomena during consultation

3 Abnormal preoccupations occupy significant proportion of subject's attention, cause significant distress to subject or practical problems for carers; eg subject won't let matter drop after an argument / keeps returning to the same issue all day.

4 Abnormal preoccupations occupy most of subject's attention and cause major problems and distress for subject and carers; may be preoccupied with the topic for several days at a time

Frequency

0 Never, or almost never

1 Seldom: <1 times a week

2 Sometimes: 1-4 times a week

3 Frequently: 5-6 times a week

4 Every day or almost every day

Delusions

I am going to ask you about unusual experiences that people sometimes have. Has it seemed like people are out to get you or perhaps controlling you? Has it seemed like you have special powers or importance or that books, TV and radio statements are referring to you? Are there any other unusual things you experience that I have not asked you about? Have you felt that people were unduly interested in you or that things were arranged to have special meaning or even that harm might come to you? Have there been any other odd or unpleasant experiences of any kind recently?

Severity

0 Symptom absent

1 Questionable or trivial

2 Overvalued ideas (not true delusions) present for part of the day; do not affect behaviour

3 Overvalued ideas present for much of day; behaves as if these beliefs true; can be persuaded with difficulty that mistaken

4 Delusions: false beliefs, held with unshakeable conviction, not shared by social / cultural group, present continuously for ≥ 7 days

Frequency

0 Never, or almost never

1 Seldom: <1 times a week

2 Sometimes: 1-4 times a week

3 Frequently: 5-6 times a week

4 Every day or almost every day

Hallucinations

Have you heard things that other people could not hear such as noises or voices of people whispering or talking? Did you ever have visions or see things that other people could not see? How about any other strange sensations in your body, skin, smell or taste? We ask this question of everyone and would like to ask you; do you ever seem to hear voices or noises when there is nobody about and no ordinary explanation seems possible, or see or feel things other people can't? What about any other unusual experiences or talents that some people have such as having second sight or being aware of strange presences?

Severity

0 Symptom absent

1 Questionable or trivial

2 Reports hallucinations when asked but do not appear to cause any distress or affect subject's behaviour; experiences hallucinations but is aware that they cannot be real.

3 Hallucinations affecting behaviour eg looking for source of voices, but not appearing to cause much distress

4 Clearly distressed by hallucinations and preoccupied with them

Frequency

0 Never, or almost never

1 Seldom: <1 times a week

2 Sometimes: 1-4 times a week

3 Frequently: 5-6 times a week

4 Every day or almost every day

Apathy

Within the last month have you ever found that you have lost interest in things that used to be important to you? Do you sit around a lot doing nothing? Are you just as interested as always in trying new things, starting new projects? If apathy and depression are both felt to be present it would be important to differentiate clearly with reasons between the two.

Severity

0 Symptom absent

1 Questionable or trivial

2 No longer tries new things; gentle prompting to initiate pastimes which usually enjoys; less effort to keep up with friends / relatives; puts off household tasks previously part of daily routine, gentle prompting to do these things

3 Overt prompting to take part in pastimes / carry out daily household tasks; little / no effort to keep up with friends, left to others to initiate social contacts; takes part in & enjoys conversation; tends to follow, less likely to initiate a change of subject

4 Performs no household tasks even if prompted repeatedly; never initiates activities, no interest in pastimes; impoverished speech, rarely initiates new conversation topics except re own needs; active choices limited to selecting TV programmes; switches on / changes channel

Appendix H Cognitive battery

Cognitive tasks were administered by the same investigator at each visit, in a single session lasting about 1½ - 2 hours. The order of tasks was kept constant. The tasks were:

- Pre-morbid IQ: National Adult Reading Test (NART) (Nelson *et al.* 1991)
- UHDRS cognitive tests:
  - Stroop colour-reading, word-reading and colour-word interference (Delis *et al.* 2001)
  - Letter fluency (Benton 1976)
  - Symbol Digit Modalities Test (SDMT) (Smith 1968)
- Other executive / psychomotor tasks:
  - Category fluency (animals) (Delis *et al.* 2001)
  - Homophone Meaning Generation Test (HGMT) (Warrington 2000)
  - Trail-Making Test A and B, A cancellation
- Memory:
  - Hopkins Verbal Learning Test-Revised (Brandt *et al.* 2001)
  - Digit span forward and backward, with 3 trials at each length (Wechsler 1981)
  - Recognition Memory Test (RMT) (Warrington 1984)
- Naming: the Graded Naming Test (GNT) (McKenna *et al.* 1983)
- Visuo-spatial:
  - Silhouette subtest of the Visual Object and Space Perception Battery (VOSP) (Warrington *et al.* 1991)
  - Benton Facial Recognition Test (Benton *et al.* 1983)

- Emotion recognition: 24 faces from the Ekman and Friesen battery were used (Ekman *et al.* 1976)

Appendix I Semi-automated segmentation of whole brain

(Adapted from Dementia Research Centre Standard Operating Procedure and reported by Freeborough *et al.* 1997)

Introduction

The aim of the segmentation is to label all voxels that are predominantly brain while excluding non-brain, for example, CSF, dura and the superior sagittal sinus. In some cases, whole brain segmentation can be accomplished in four steps:

- Intensity thresholding to exclude voxels outside the intensity range for brain.
- Erosion to break connections between brain and non-brain.
- Dilation to restore eroded brain.
- Rethresholding to include missing voxels within brain.

NB: All steps operate on all slices in a three-dimensional fashion. At each stage, the current segmentation is reviewed through the whole volume.

Intensity thresholding

Intensity thresholds are set to exclude voxels brighter than brain (e.g. scalp) and darker than brain (e.g. CSF).

The upper threshold should be set first. Anything brighter than the upper threshold will be excluded. Leaving the lower threshold on zero, increase the upper threshold until there is no green signal intensity within brain.

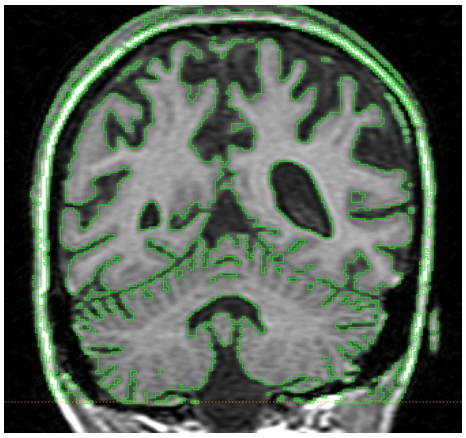
Secondly, the lower threshold is set initially to lower than half the value of the upper threshold and then adjusted to include as much of brain as possible while minimising the inclusion of non-brain. The green intensity region should include most of the brain.

The area around the cerebellum and temporal lobe are checked to ensure that most of the bridges — signal intensity connections linking brain to dura or superior sagittal sinus — are broken.

Ideally the lower threshold should be taken to a high enough value so that in the next step only one erosion is needed to remove non-brain.

Finally, the axial cut-off slice is selected. This removes any structures below the inferior edge of the cerebellum. The coronal slice in which the cerebellum extends most inferiorly is the cut-off. Other slices are checked to ensure that all cerebellum is included.

After 'Accept' is pressed, MIDAS searches for the largest connected three-dimensional object in the current region (i.e. brain) and removes any smaller unconnected objects.



Thresholded image with voxels lighter and darker than brain excluded, and with the axial cut-off slice set to the lower extent of the cerebellum

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Erosion – breaking remaining connections between brain and non-brain

The erosion removes a layer of edge-voxels from all surfaces of the region. The purpose is to break thin connections between brain and scalp. After each erosion, the program retains only the largest connected region. The minimum number of erosions necessary to isolate brain from non-brain are used. The usual range of erosions is 0-3. High numbers of erosions remove significant quantities of brain from the region that cannot be recovered in the dilation stage. In this case, the thresholds may be at fault or the image may be artefactual or noisy.

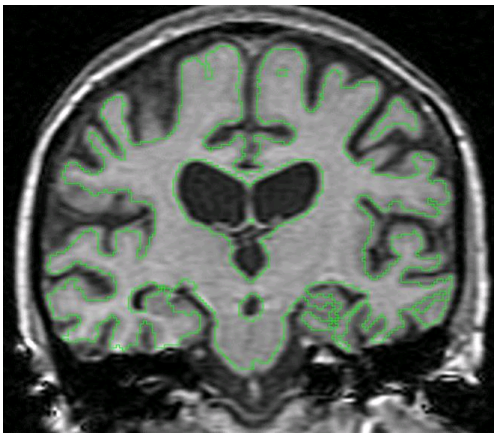
Eroded voxels have a signal intensity below the upper threshold value.

The number of erosions is increased one at a time until all non-brain structures are removed. If the edges of the brain region are still within grey matter, erosions can be increased by one. If the edges

of the brain region are within white matter, another erosion may remove too much. There are two ways to proceed.

- Often the brain remains connected to scalp on only a few slices, often around the temporal lobes. If connections can be identified visually, then they can be broken interactively at this stage by striking through them with the mouse while holding down the middle button. Areas where connections have been interactively broken appear orange. When all connections between brain and non-brain have been removed, all parts of the region external to brain are removed automatically. The width of the interactive connection breaker can be set by adjusting the cursor width slider bar from 1-6. The right mouse button will remove the connection breaker and the left will add in additional region.

- Further erosions can be performed, but the upper threshold can be lowered so that the white matter of brain is not eroded. This can break lower intensity connections between brain and scalp or dura without eroding any more brain.



Eroded region showing disconnection from non-brain tissue

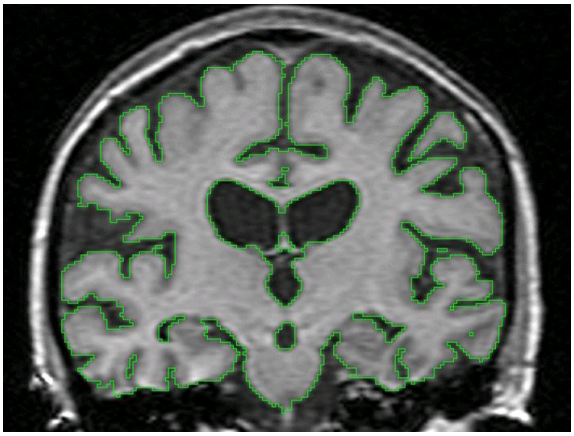
Dilation – restoring eroded brain

The purpose of this step is to recover the brain voxels that have been removed by erosion. Each dilation grows out from the edges of the current brain region but only into voxels within the currently defined range of intensities that represent brain. The intensity thresholds that appear at this point are automatically set by calculating the mean signal intensity over the whole brain region taking 60% and 160% of this value. These percentages have been determined to be suitable for T1-

weighted volumes. These thresholds are re-calculated after each dilation as more voxels are recovered.

The number of dilations that are needed to recover all brain voxels depends on the number of erosions (typically number of erosions plus 1 or 2). The number should be kept low enough so that no dura or sagittal sinus are included even if this results in a small proportion of brain not being included. Regions around the eyes are checked to ensure that the region has not extended into this area.

The connection breaker can also be used at this stage to remove any final areas of scalp / dura which are attached.



Dilated image showing disconnection from non-brain tissue

Rethresholding – reclaiming remaining excluded areas

This step is generally not required. However if there are small areas missing from the brain region this may remove the need for manual editing. It will also remove any tissue outside the thresholds within the brain. This step completes the segmentation. The rethresholding box is a box of voxels that moves across the outlined brain region and fills in any area within that region that has been omitted by step 3 subject to the following constraints:

- The omitted area must be smaller than the box.
- Only those voxels that have a signal intensity of between 60% and 160% of the mean brain intensity will be included.

- The missing area must already be internal to the region.

Generally, a rethresholding box size of 6 will be large enough to recover any areas that have previously been excluded.

Manual editing

In cases where either some non-brain tissue remains in the segmentation, or brain tissue has been omitted, manual editing is required. Unlike the automated whole brain segmentation, editing is limited to the slice being viewed — manual editing does not work on the 3D volume.

The brain region generated by the automated segmentation process above is highlighted (which should turn from blue to red), and manual editing mode is activated. Another window gives any one of two orthogonal views to that in the main window. Slices where the segmentation needs editing are selected, with particular attention to the temporal lobes and cerebellum, as these are areas where editing is most often required. If brain tissue has been excluded, the border can be deleted and a new border redrawn.

New regions can be included by placing a seed in the appropriate area. Either a border can be drawn manually around the structure, or alternatively upper and lower thresholds can be selected to delineate the structure automatically.

Finally, the new region is viewed in all planes to ensure that the manual editing process has not introduced new errors into the segmentation. The region is saved in the database.

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